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RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES

INCLUDING

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INTERNAL MEDICINE, PHYSIOLOGY, PSYCHIATRY, SURGERY,
AND VETERINARY MEDICINE,

(PROJECTS AND WORK UNITS ARE
LISTED IN TABLE OF CONTENTS)

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FORWARD

IN CONDUCTING THE RESEARCH DESCRIBED IN THIS REPORT, THE INVESTIGATORS ADHERED TO THE "GUIDE FOR THE CARE AND USE OF LABORATORY ANIMALS" AS PROMULGATED BY THE COMMITTEE ON CARE AND USE OF LABORATORY ANIMALS OF THE INSTITUTE OF LABORATORY ANIMAL RESOURCES, NATIONAL ACADEMY OF SCIENCES - NATIONAL RESEARCH COUNCIL.

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SUMMARY

THE VARIOUS SUBJECTS COVERED IN THIS REPORT ARE LISTED IN THE TABLE OF CONTENTS. ABSTRACTS OF THE INDIVIDUAL INVESTIGATIONS ARE INCLUDED ON THE DD FORM 1498 INTRODUCING EACH WORK UNIT REPORT, AND NAMES OF THE INVESTIGATORS ARE GIVEN AT THE BEGINNING OF EACH REPORT.

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Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 001 Epidemiologic Studies of military diseases

Investigators.

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1. Febrile Illness at Fort Campbell, KY

Following jungle training at Fort Sherman, Canal Zone, in October 1977, 12 members of a 35-man engineer platoon were hospitalized with a febrile illness (see Walter Reed Army Institute of Research FY 78 Annual Progress Report work unit 001 "Epidemiologic Studies of Military Diseases"). The illness was characterized by headache (100%), malaise (100%), myalgia (92%), chills (84%), arthralgia (84%), and nausea (67%).

Serological results done at the Center for Disease Control on sera collected in December indicated that eight of the twelve hospitalized patients gave a positive "M precipitin" reaction and also had CF antibodies for histoplasmosis. The results confirmed that the hospitalized patients had recent infection with histoplasmosis.

Epidemiologic investigation suggested that the engineers were probably exposed during a civic action project when they cleared vines and caves at Fort San Lorenzo.

A survey of those persons who were involved with the jungle training and who were still available was done in December in an attempt to determine if the engineer platoon was uniquely involved. A sample of those surveyed was tested for histoplasmosis antibodies and the results are presented in the table below.

ANTIBODY PREVALENCE TO HISTOPLASMOSIS IN SAMPLES FROM VARIOUS GROUPS WHO UNDERWENT JUNGLE TRAINING IN PANAMA

Group	# Studied	# Positive	%
Ft. Campbell sick engineers	12	8	67
Ft. Campbell not sick engineers	18	3	17
Ft. Campbell not engineers, not sick, Co. C.	20	1	5
Ft. Campbell not engineers, not sick, not Co. C	13	0	0
Ft. Bragg sick	5	0	0
Ft. Lewis sick	13	0	0

In summary, an outbreak of histoplasmosis resulted in the hospitalization of 12 men with a non-specific febrile illness. Although the paucity of pulmonary signs and symptoms in the group suggested that histoplasmosis was an unlikely diagnosis, subsequent serologic testing confirmed the diagnosis.

This study is now complete and a manuscript is in preparation.

2. Hepatitis in the 25th Infantry Division, Schofield Barracks, Hawaii

A thorough discussion of the background and initial findings in this study is contained in the WRAIR annual report of 1977-78 Work Unit 001. Work since this initial report has involved refinement of the confirmed case group.

CASE DEFINITION

A presumptive case was defined as an individual associated with Quad D showing an SGOT value ≥ 80 units (normal ≤ 40) on the SMA-12/60 autoanalyzer and no other non-viral or extrahepatic reason for the elevation. Of the original twenty-five cases described in last year's report, 19 presumptive acute hepatitis cases were defined in Quad D. Sera from all presumptive cases were checked for IgM antibody to hepatitis A virus at the WRAIR Department of Virus Diseases using a solid-phase radioimmunoassay of polyvinyl microliter plates coated with goat anti-human IgM antisera. The confirmed case group was defined to include only those presumptive cases with IgM antibody. Using this definition, 16 of 19 presumptive cases were confirmed. The number of confirmed cases became 17 after a control was found to have IgM antibody to HAV when studies were performed at WRAIR following the on-site investigation. Although no clinical data is available on this latter individual, the remaining 16 confirmed cases were interviewed and questionnaires dealing with clinical and epidemiologic factors related to hepatitis were administered.

SUSPECT SOURCE IDENTIFICATION AND EVALUATION

The epidemic curve was consistent with a point source epidemic. Although questionnaires failed to yield suspect common activities, habits, or contacts to explain the outbreak, multiple subjective complaints concerning Quad D mess hall sanitation and the Kahuku training area water supply, made these the two primary areas of investigation. Thorough examination of the mess hall personnel, facilities, and practices produced only one deficiency: the kitchen ice server was found to contain ice positive for fecal coliforms.

The Kahuku training area investigation produced evidence of several deficiencies. The hypochlorite injector which treated the field water point worked sporadically and the water had been declared unpotable in August of 1977. Questioning of the Quad D mess sergeant disclosed that he had utilized this water without supplemental chlorination during field exercises immediately prior to the outbreak. Investigation of the water holding tank for this training area revealed

the inspection hatch had been broken, and culture of the water grew mammalian coliforms. A record review revealed that the latest chlorine residual, measured on 11 April, was zero.

HYPOTHESIS TESTING

The two hypotheses tested were that either the D Quad mess hall or the Kahuku field water point was the source of this hepatitis outbreak. To test these hypotheses, two controls were chosen for each confirmed case and matched for age, sex, rank, and unit. Questionnaires dealing with exposure to the D Quad mess hall and the Kahuku training area were administered to both cases and controls. Controls were excluded from the analysis if they failed to appear for interview or their sera contained IgG antibody (thus denoting lack of susceptibility). This exclusion process left 20 of the original 32 matched controls. Fourteen of these could be matched with cases and utilized in the matched pair analysis shown below:

MESS HALL

	Cases (N=17)			Controls (N=20)			
Usually Eat	+	-	%+	+	-	%+	%Difference
Brkfst	9	8	53	7	13	35	18
Lnch	14	3	82	7	13	35	47
Dnnr	11	6	65	7	13	35	30
Brkfst+Lch	9	8	53	6	14	30	23
Lnch+Dnnr	11	6	65	7	13	35	30
Brkfst+Dnner	8	9	47	6	14	30	17
B+L+D	8	9	47	6	14	30	17

KAHUKUS

	Cases			Controls			% Difference
	+	-	%+	+	-	%+	
Any time	15	2	88	19	1	95	-7
April	12	5	71	15	5	75	-4
May	8	9	47	12	8	60	-13
June	12	5	71	15	5	75	-4
Apr+May	6	11	35	10	10	50	-15
May+Jun	8	9	47	10	10	50	-3
Apr+Jun	9	8	53	11	9	55	-2
A+M+J	6	11	35	9	12	40	-5

MESS HALL EXPOSURE

1 Meal	Control		Breakfast	Control	
	+	7 4		+	4 3
Case			Case		
	-	3		-	2 5
2 Meals	Control		Lunch	Control	
	+	6 4		+	6 5
Case			Case		
	-	4		-	3
3 Meals	Control		Dinner	Control	
	+	3 3		+	6 3
Case			Case		
	-	2 6		-	5

The importance or risk of the exposure is determined by dividing the upper-right hand corner of each 2x2 table by the lower-left-hand corner. A quotient of >1 indicates the exposure increased the risk of disease. An identical analysis was done for exposure

to the Kahuku training area and, unlike the mess hall data above, this yielded no quotients >1. To use all valid case-control data, an unmatched, pooled analysis was done.

POOLED ANALYSIS - UNMATCHED DATA
MESS HALL

Cases (N=17)				Controls (N=20)			
Ever Eat	+	-	%+	+	-	%+	%Difference
Any Meal	16	1	94	17	3	85	9
Breakfast	15	2	88	17	3	85	3
Lunch	15	2	88	11	9	55	33
Dinner	13	4	76	11	9	55	21
Brkfst+ Lnch	14	3	82	11	9	55	27
Lnch+Dnnr	12	5	71	10	10	50	21
Bkfst+ Dnnr	13	4	76	11	9	55	21
Bkfst+Lch +Dnnr	12	5	71	10	10	50	21

The cases demonstrate, by their percent positive responses, a greater exposure to the Quad D mess in both the "ever eat" and "usually eat" categories. Here, comparing the differences in "% positive" responses between cases and controls, the % differences are greater in the "usually eat" group than in the "ever eat" group, consistent with an increased risk with increased exposure (dose-response). Data in this pooled analysis agrees with the matched pair results above by showing exposure to the mess hall increased the risk of hepatitis.

Data on exposure to the Kahuku training area in the pooled analysis agree with matched pair results, minimizing this as a risk factor. The exposure again seems

to be greater for controls than for cases. If exposure was related to the Quad D dining facility, the training (and eating) in the field may have, indeed, been protective. The large negative "%difference" value for the May exercise (which was closest to the appropriate exposure time, given that the median case had onset of symptoms on 16 June) suggests this possibility.

DISCUSSION

Despite considerable evidence incriminating the Kahuku field water point, hypothesis testing points to the mess hall as a more likely source. Certainly, the presence of coliforms make the ice dispenser suspect. With this as an indicator of poor mess hall technique, an ill foodhandler could have unknowingly contaminated the eating area and recuperated by the time of the EPICON Team's investigation. Clearly, both the dining and training facility were in need of improvements, and the investigation served to highlight these areas.

An extensive urine screening of units residing in Quad D was conducted to detect subclinical or incubating cases of Hepatitis A. This screening, which employed the Multistix and Chemstrip 8 dipstick techniques, was performed on 1835 troops and yielded only one subsequently confirmed case. This individual was already symptomatic and planning to seek medical attention at the time he was screened. Because this screening was planned and executed hurriedly to meet operational requirements, there were many errors in technique and execution. To definitely establish whether testing for urine bilirubin has any role as a screening tool for hepatitis A would require carefully controlled and standardized future studies.

The use of new techniques to determine the presence of IgM antibody to Hepatitis A virus in order to refine both the case and control grouping was of great importance in this investigation. A more thorough discussion of the technique can be found in the section of this report contributed by the Department of Virus Diseases, Work Unit 135, "Mechanism of Transmission of Hepatitis Viruses." Adaptation of this technique for on-site field use will greatly enhance future EPICON hepatitis A outbreak investigations.

3. Tuberculin Skin Test Reactors Fort Knox, KY

In the Spring of 1978, 2939 school age children were tuberculin skin tested at Fort Knox, KY. This represented three-fourths of the school population on post. The positivity rate in the Spring of 1978 was 1.63% as compared to previous rates of 0.14% in 1976 when 1st, 5th, and ninth graders were skin tested, and 0.46% for 1977 when the entire school population of about 4000 was tested. EPICON assistance was requested to uncover the reason for the apparent increase in positive tuberculin skin test reactors (1978 WRAIR Annual Progress Report, Work Unit 001). An extensive investigation was conducted but no source of infection could be found. Retesting of the entire school age population was recommended to provide a better estimation of the amount of exposure and conversion occurring.

Retesting of this population was done in the Fall of 1978. 4087 school age children were tuberculin skin tested. Thirty-six (36) reactors were discovered giving a positivity rate of 0.88%. In the Spring of 1979, 1340 children in this population were again tested, identifying five (5) positive reactors. The positivity rate was 0.37% for this testing.

TST - School Population - Ft. Knox - 1978/1979

Time	# of Students Tested	# of Students Pos.	% Pos.
Spring 1978	2938	48	1.63
Fall 1978	4087	36	0.88
Spring 1979	1340	5	0.37

In addition to students, testing was performed on all persons involved with the schools. No active disease was found. An increased awareness of tuberculin skin test reactors throughout the health system at Fort Knox is one outcome of this investigation. Data on results of TST given by other clinics is being closely monitored.

Consequently, there has been an increase in the number of adults (excluding basic trainees) being placed on prophylactic treatment. Exposure is probably due to various sources and occurs in several different

places. The Fort Knox population is a transient one as are most military populations. Several TST reactors had recently arrived at Fort Knox from other areas. Those that had definitely converted while at Fort Knox could have been exposed in other areas such as summer vacation trips, and off post activities. In the subsequent testing in the fall of 1978 and the spring of 1979 consistent high rates of positivity for schools were not seen. Mudge and Stevens schools had lower rates, while some of the other schools which had low rates in spring 1978, had high rates fall 1978 and low rates spring 1979. This suggests that the screening programs were culling out positive reactors and that the conversion rate was reasonably low.

4. Leishmaniasis Among Troops Undergoing Jungle Training in Panama

This study was initially described in the FY 77 Walter Reed Army Institute of Research Annual Progress Report Work Unit 001 "Epidemiologic Studies of Military Diseases."

Briefly, a battalion of the 82nd Airborne Division from Fort Bragg, North Carolina was studied pre- and post-deployment to the Jungle Operations Training Course at Fort Sherman, Canal Zone. They were examined for clinical as well as serological evidence of leishmanial infection.

Aspirates of suspect lesions were collected from 30 individuals. They were cultured and ten were positive for leishmanial organisms. The overall attack rate was 1.6/100 men deployed. By unit the highest rate was in B company (4.1/100 men).

Using a modified indirect fluorescent antibody test only 1 of the 10 culture positive cases showed sero-conversion. Twenty-two percent of 610 paired sera were positive, but loss of positivity in 70 pairs pre- to post-deployment suggested that the test was not specific for leishmanial antibody.

A manuscript is in preparation.

5. Determination of Congenital Malformation Rates for Seven U.S. Army Posts

This study was previously described in the FY 77 and FY 79 Walter Reed Army Institute of Research Annual Progress Report Work Unit 001 "Epidemiologic Studies of Military Diseases." The study is now complete and a final report has been written.

Two conclusions could be drawn from the data:

1) the rates for infants born at the Fort Hood hospital with Category III congenital malformations, when compared to the other post hospitals studied, did not appear to have been excessive or subject to wide variation.

2) the rates for Fort Campbell hospital may require further investigation since there is a consistent upward trend in all three classes of malformations.

No comparisons between Army dependent populations and civilian populations can be made since civilian populations were not studied. CHAMPUS records were not examined, therefore, data were incomplete. For example, at Darnell Army Hospital (DAH), Fort Hood, in 1974 1.2 CHAMPUS nonavailability certificates were issued for each live birth at DAH. Additionally it is not known what variables differentiate the CHAMPUS population from the post hospital population; therefore, direct comparisons between rates of congenital malformations at different Army hospitals may result in invalid conclusions.

6. Description of Renal Disease for Active Duty Personnel

This study originally described in the FY 77 Walter Reed Army Institute of Research Annual Progress Report Work Unit 001 "Epidemiologic Studies of Military Diseases" has been transferred to the Uniformed Services University of the Health Sciences School of Medicine.

7. Description of the Army's Experience with Guillian-Barre Syndrome

This study was reported in the FY 77 and FY 78 Walter Reed Army Institute of Research Annual Progress Report Work Unit 001 "Epidemiologic Studies of Military

Diseases." The study has been completed and the manuscript is in final draft.

8. System Analysis of a Fort Dix General Practice Clinic

Analysis of data from a test morbidity surveillance system implemented by Preventive Medicine residents Major H. M. Scagliola previously reported in FY 78 WRAIR Annual Progress Report Work Unit 001 was concluded with the following findings and recommendations:

The questionnaire used to collect data was effective and well accepted by patients and physicians. Implementation of a similar system elsewhere will be facilitated if principles of communication and human behavior are used when introducing change into the existing situation, and if computerized data processing is available. The information gained through such a surveillance system should aid in obtaining demographic information on patients, and in maintaining epidemiological surveillance of disease.

9. Investigation of an Outbreak of Hepatitis A at SHAPE, Belgium

On 14 Feb 79 an EPICON team traveled to SHAPE, Belgium to investigate an ongoing hepatitis outbreak. Twenty-two cases had occurred prior to the team's arrival and the total rose to 45 by the end of March. The diagnosis was confirmed in the WRAIR Laboratories as hepatitis A. An active search was made for old and new cases, and a central registry and information center was established. All cases and family contacts were bled and interviewed. Every case who did not have close prior contact with another case was found to be closely associated with either the nursery or the preschool. Some were students, some were parents of students and one was a member of the nursery school staff. Examination of school records revealed an increased absentee rate in the preschool since the Christmas vacation. The preschool itself was understaffed (30:1 student/teacher ratio) and there were major sanitation deficiencies in the bathrooms. The nursery was adequately staffed but had only 2 toilets for up to 60 children and 6-10 staff members. Aside from the schools, there were no other common factors identified: water

purity was satisfactory on numerous tests; there were no large gatherings or parties attended by most cases; places of residence were scattered throughout the Mons - SHAPE area; and no common food vehicle, such as shellfish, was identified. Recommendations included ISG administration to case contacts, students, staff and family members of students attending the nursery and pre-school; and increasing the staff size of the pre-school and the Preventive Medicine activity.

10. Outbreak of Tuberculosis in the Monkey Colony at the Division of Neuropsychiatry, WRAIR

On 13 June 1979, a monkey being used by the Division of Neuropsychiatry, WRAIR, was found dead in its cage. An autopsy revealed gross and microscopic evidence of miliary TB, evidence which was later confirmed by culturing the organism from the lesions. Skin testing of the colony revealed two additional infections. Both animals were sacrificed. The Division of Preventive Medicine was asked to assist in the control and investigation of this epidemic.

Since TB in monkeys is a rapidly fatal disease and these monkeys had not been exposed to new monkeys for over a year, the source of infection was probably a human. Control measures were designed to minimize man-monkey contact and reduce the possibility of disease spread by fomites. Potentially contaminated areas were cleaned and decontaminated. Monkeys were confined to their rooms and access to these rooms was limited to essential personnel. All personnel handling monkeys wore masks, caps, and gowns.

The three affected monkeys came from three different rooms. These rooms were inspected and the details of daily routines were noted. All monkeys were skin-tested every two weeks until all were negative on three consecutive tests. All identifiable human contacts including casual contacts, were screened using intradermal PPD, chest X-ray, or both. No further cases occurred in the monkey population. All were skin test negative on July 2, 16, and 30. Culture results from autopsy material taken from the two skin test positive monkeys were negative. The inspection of the facilities revealed abundant opportunities for man-monkey contact. Office and computer areas were immediately

adjacent to monkey cages and were served by ventilation systems whose main purpose appeared to be rapid transport of microorganisms from monkey to man. Sixty-four persons, including all those with significant monkey contact, were screened for tuberculosis. Forty-seven (73%) of these had a negative PPD. Fourteen (22%) had had a positive PPD in the past and had a normal X-ray at the time of the investigation. Two persons converted from a negative to a positive PPD, but both had normal chest X-rays and were considered unlikely to have infected the monkeys. They were referred for further workup and treatment. One person had been treated for TB in the past, had questionable X-ray findings, reported symptoms consistent with TB lasting a month and had at least some contact with all three simian cases. However, three sputum specimens produced in the normal manner and one after 20 minutes on the IPPB machine were negative on both acid-fast stain and culture. His symptoms have since disappeared in the absence of any specific therapy. Thus the outbreak ended and no index case was identified. Recommendations were made to reduce the chance of further outbreaks.

11. Epidemiology of Arboviral Infections Along the Transamazon Highway in Brazil

As reported in WRAIR Annual reports 1974-1976 Work Unit 001, an epidemiology surveillance program was initiated along the Transamazon Highway in January 1974, in the state of Para to study disease incidence in the newly arrived colonists. Locally trained interviewers visited a stratified random sample comprised of 20% of the roadside colonists outside the towns of Maraba and Altamira. The distribution of the population by age, sex and state of origin was similar to that of the entire population as reported by INCRA (Instituto Nacional de Colonizacao e Reforma Agraria). Blood was drawn every six months and whenever a colonist reported a febrile illness on one of the routine 2 week visits. Examinations performed on the samples included thick and thin smear for malaria, FA and CF tests for selected bacteria and parasites and HI tests for arboviruses using 19 viral antigens known to exist in the area. The results from these tests have been coded for computer analysis and will be included in a manuscript which is being prepared for publication.

12. African Trypanosomiasis and Leishmaniasis in Kenya

A site visit was made to the WRAIR SFA in Kenya to determine the potential for expanding the laboratory's current work on African Trypanosomiasis.

Several trips were made to field sites in the Lambwe Valley and in the Machakos area. USAMRU-K data were reviewed and disease problems were discussed with USAMRU-K personnel and Kenyan health officials. The entire Lambwe Valley was mapped, each house enumerated and the population of each area estimated. A serum survey including all children and many adult volunteers turned up only two cases of trypanosomiasis, one a relapse, in the peak transmission season. A review of the USAMRU-K records revealed a decreasing or stable incidence, and it was decided that there simply were too few human cases to justify an expansion of effort in that area.

Leishmaniasis presented an entirely different situation, however. Several cases were seen in Nairobi hospitals and special wards were devoted solely to the care of leishmaniasis patients in Machakos and Makuemi Boma. Little research had been done recently despite a large increase in cases seen in local clinics. Data from the 1940's had implicated dogs as the reservoir and *Phlebotomus martini*, a sandfly often found in termite mounds, as the vector. However, a recent survey of 1000 dogs had resulted in only two with the disease and in our 3 day trip we saw only one dog and no termite mounds at all. It was recommended that the work of USAMRU-K be expanded to include the study of leishmaniasis, especially a search for vectors and reservoir hosts and an evaluation of current treatment regimens.

13. Adenovirus Antibody Prevalence in Recruits

In early 1976, the prevalence of antibody to selected adenovirus types was measured in personnel arriving for Basic Combat Training (BCT) to define the potential impact of these strains on respiratory disease rates in trainees. The design, methods and conclusions of these studies have been described previously in the WRAIR Annual Reports.

The demographic and questionnaire data collected during these surveys were reviewed in depth. This was the final review of the adenovirus prevalence data, and a manuscript is in preparation for submission to the American Journal of Epidemiology. The percent of trainees with antibody to adenovirus #7 was significantly lower at Fort Leonard Wood. The reason for this is unknown and may simply represent chance variation that is to be expected when many variables are studied. The samples were also studied by comparing posts to refine sampling techniques if necessary. Trainees sampled at the four posts did not differ significantly by age, marital status, household size, years of education, polio virus 1,2,3 titers and the proportion of recruits with prior basic training. There was evidence for differing regional composition reflecting increased input of personnel from neighboring areas to the particular BCT Post.

14. Dengue Fever Outbreak in the Bahamas 1977

The Dengue fever outbreak that occurred in the Bahamas in 1977 was reported in the FY 78 Walter Reed Army Institute of Research Annual Progress Report Work Unit 001 "Epidemiologic Studies of Military Diseases." There are several aspects of the investigation that are still in progress.

Clinical Description: Definitive diagnosis of Dengue Type I was made on over fifty patients by virus isolation. A check list of clinical signs and symptoms are available on these individuals. The data are presently being analyzed to produce a clinical picture of Dengue Type I in this population. The clinical presentation will also be analyzed by age.

Apparent/inapparent ratio: Approximately 200 Bahamian police submitted blood specimens in Nov 1977 and again in Jan 1978, and completed questionnaires about illness and symptoms. The sera have been tested at the University of the West Indies and at WRAIR for antibody to Dengue Type I virus. Analysis of this data will provide information on attack rates and apparent/inapparent infection ratios.

Risk factors of infection: During the household survey a number of variables were tabulated including: day-time activity, window screening, larval breeding sites, travel, age, sex, number in household, etc. These will be correlated with reported disease in the individuals and the households.

A manuscript dealing with the investigation has been published in the scientific literature and is listed at Literature Cited.

15. Simultaneous Administration of Live, Enteric-Coated, Oral Adenovirus Types 4,7, & 21 Vaccines: Safety, Efficacy, and Immunogenicity

The design and execution of this study is described in the WRAIR Annual Report covering the 1976-77 period. Basically, the study involved the selection in October, 1976 of 600 Basic Training volunteers at Ft. Dix, New Jersey, 300 of which received adenovirus 21 vaccine (ADV-21) in conjunction with vaccines for adenovirus 4 and 7 (ADV-4, ADV-7), and 300 of which received placebo with the 4 and 7 vaccines. The safety, efficacy, and immunogenicity of the ADV-21 vaccine was to be determined by drawing pre- and post-immunization sera from both groups to check for antibody response and by following all subjects for hospitalizations during the 2 month study period. The testing of sera for neutralizing antibody to all ADV types was interrupted because of problems with the primary human embryonic kidney cell culture line. This year, a substitute microneutralization technique was developed and processing of specimens continued.

A random sample was selected containing 169 individuals, 89 in the vaccine group and 80 in the placebo group. Sera from these individuals have been tested for neutralizing antibody pre- and post-vaccination. Identical studies have been performed on acute and convalescent sera from the hospitalized patients.

Presently, with the assistance of the Department of Biometrics, all data in this study are being computerized so that an analysis can be performed and a final report written.

16. Morbidity and Mortality Resulting from Motorcycle Accidents in the U.S. Army

This study was briefly described in the FY 78 Walter Reed Army Institute of Research Annual Progress Report Work Unit 001 "Epidemiologic Studies of Military Diseases." Analysis of the IPDS data tapes is still in progress. All hospitalizations with a cause of injury code of motor vehicle accident from 1973 to 1978 are included on the tape. One finding of interest is that 27.6% of all patients hospitalized for motor vehicle accidents were operators or passengers of motorcycles or mopeds.

17. A Toxoplasmosis Outbreak Occurring in U.S. Soldiers Undergoing Jungle Training in the Canal Zone

Within two weeks of returning from a three week jungle training course at Fort Sherman in the Canal Zone, 30 of 88 soldiers from the Combat Support Company of the 1/508th, 82nd Airborne Division, Fort Bragg, experienced a febrile illness. The illness was characterized by fever, chills, headache, myalgia, malaise, and lymphadenopathy. Laboratory studies revealed elevated SGOT, LDH and alkaline phosphatase values. Total white counts were normal, although most had atypical lymphocytes on peripheral smear. Most experienced an illness of flu-like severity although three were hospitalized. All have recovered with sequelae.

Acute and convalescent sera on 12 individuals and late sera on another 18 were tested at the Center for Disease Control and demonstrated either seroconversion or high titers of IgM when tested for toxoplasmosis using the IFA and IHA tests.

Serological survey of the entire involved company demonstrated evidence of recent infection in only two persons who were not clinically ill. Thus over 90% of those infected were clinically ill. All but one of the cases had at least two abnormal liver function tests with their SGOT and LDH values in the 300-400 range six weeks after acute illness.

Epidemiologic investigation suggested the exposure to the Toxoplasma gondii organism occurred during the four day field exercise portion of the jungle training

course. This would indicate an incubation period of 5 to 18 days with a median of 12 days.

No meal, food source, or soil exposure could be implicated in the transmission. Epidemiologic evidence pointed to a water point as the most likely source of exposure. Presently water from the water point is being processed in an attempt to isolate toxoplasma oocysts.

Also presently in progress is a serologic survey of 60% of the 4/10 Battalion that is stationed at Fort Davis, Canal Zone. These troops have frequent exposure to the jungle. A cross-sectional study comparing time in Panama and prevalence of toxoplasma antibody will help determine whether the outbreak was a unique occurrence, or something that has previously occurred but gone unrecognized.

18. Leishmaniasis Experience of the Middle America Research Unit

The leishmaniasis projects, Prevalence of Leishmaniasis in U.S. Army Troops (1971-1977), and Leishmaniasis Among Troops Undergoing Jungle Training in Panama, reported in the FY 78 and FY 79 Walter Reed Army Institute of Research Annual Progress Report Work Unit 001, highlighted the lack of data on the epidemiology of leishmaniasis and the natural history of the disease particularly in persons with short term exposure. In an attempt to develop further information about leishmaniasis, data from the Middle America Research Unit (MARU), Canal Zone is being analyzed.

During a period of approximately 10 years data were collected on 151 military patients treated at the MARU for clinical leishmaniasis. Demographic, clinical, laboratory and therapeutic data are included. Although there were problems in standardization of the questionnaires useful information is available.

19. The Design and Institution of a Morbidity Reporting System for the 101st Airborne Division at Fort Campbell, KY

INTRODUCTION

To understand and analyze the medical needs of a population, outpatient information must be collected

and reported, since only a small percentage of all patients are hospitalized. Currently, such outpatient morbidity information is not collected in the Army and, therefore, is not available for epidemiologic research or disease outbreak investigations. Since the WRAIR Division of Preventive Medicine is concerned with both of these latter areas, the establishment of outpatient morbidity surveillance reporting is considered important.

At the request of the 101st Airborne Division Surgeon, LTC Robert Kreutzmann, an EPICON team was dispatched to Ft. Campbell on 5 March, 1979 to design an illness/injury reporting system for the line units. LTC Kreutzman's need at the division operational level was for accurate and timely information on illness in troop units in order to advise commanders concerned with deployment readiness. In conjunction with his General Preventive Medicine Residency Training, CPT R. Prier was tasked to design and implement the system.

A REPORTING SYSTEM DESIGN

Initially, the existing medical care delivery system and reporting procedures were evaluated. Recording and reporting procedures in Aid Stations and Troop Medical Clinics (TMC's) varied greatly. Information collected on each patient upon entering a TMC or Aid Station was recorded on a log sheet but was not standardized. Information on medical problems was limited to chief complaint and not diagnosis. Demographic information was scanty or non-existent. Aid Station morbidity reporting consisted of the monthly Command Health Feeder Report, with diagnostic information limited to counts of diseases seen by general organ system category (e.g. EENT, GI, URI, Skin, etc). The TMC monthly report consisted of the Medical Summary Report, providing numbers of patients seen without reference to diagnosis.

Given the variable diagnostic information reported, and the differing formats used to submit reports, standard log-in forms and procedures, plus reporting forms and procedures, were developed and implemented for both TMC's and Aid Stations in three phases. The first phase was the already-described examination and description of existing reporting/recording procedures.

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During this phase enlisted staff, physician assistants, physicians, and the Division Surgeon's staff were requested to give their input as to deficiencies in the existing system, and to suggest what features in a new system would meet their needs. The second phase involved a general briefing of all TMC and Aid Station personnel as to the proposed design and operation of the new recording/reporting system. In addition, 3 TMC's and 3 Aid Stations were selected for a three-day trial of the forms and procedures. The third and last phase consisted of the implementation of the system throughout the 101st Airborne Division. In conjunction with the final phase, a detailed SOP was written explaining the forms and procedures and distribution to all personnel involved.

In order to gather hospital-generated patient-care information, a meeting with the hospital commander and nursing chiefs was held. Emergency Room and hospital-admission information collection procedures for active duty patients were designed and implemented. To avoid having to collect information from the complex hospital outpatient clinic system, Division personnel going to such clinics are required to log in first at TMC's and Aid Stations, with the name of the clinic entered as the diagnosis.

FIELD REPORTING

Injuries and illness which occur in the field environment are recorded using log-in and reporting procedures identical to those in garrison. During the design phase of the system, an infantry battalion deployed to the field and utilized the new Forms and procedures without difficulty.

DATA PROCESSING

Aid Station and TMC log sheet information, covering the period from Friday noon until Friday noon is transferred to a reporting form and submitted to the Division Surgeon's Office (DSO). The DSO consolidates this information, along with Emergency Room and hospital admission data, and converts it to rates for each unit by sex and diagnosis. These rates are transferred to a Unit Surveillance Form (one for each battalion-size unit) where they can be followed over time. In addition

to rates, the absolute number of hospitalizations and quarters/profiles can be followed over time. Unit strength denominator data is obtained from the Division G-1 on a regular basis.

Analysis of data from field exercises is done utilizing a separately designed surveillance form which utilizes the person-days denominator concept to allow for variable unit size and exercise duration. This permits medical and operational planning, plus comparison of unit performance in similar exercises.

FEEDBACK

Once data input has been accomplished and analysis completed, a feedback form is completed and sent to commanders and their medical staff. Four separate forms are prepared, one each for infantry, artillery, aviation, and support units of the 101st Airborne Division. This allows for comparison of illness experience in units with similar operational and training requirements.

FOLLOW-UP PROBLEMS AND FUTURE SOLUTIONS

The major flaw in the surveillance system described above is that the compilation, tabulation, and analysis of data are done manually. In late June, CPT Prier returned to Ft. Campbell to monitor progress/problems with the system. As expected, manually processing data involving multiple variables on large numbers of patients is tedious, and was taking up to two hours per day in some TMC's. The problem is compounded by a shortage of enlisted personnel at Ft. Campbell. Thus, the manpower to perform extra administrative tasks such as morbidity surveillance is limited. Therefore, because of critical shortages of physicians and support personnel, the data gathering and analysis functions failed. Although temporary measures to decrease analytic requirements were instituted, the problems of time limitations and personnel shortages still exist.

Clearly, automated data processing (ADP) would alleviate the present difficulties at Ft. Campbell and would also be essential if such a system were implemented Army-wide. An investigation of local computer equipment at Fort Campbell disclosed that no ADP resources are avail-

able for such a system. Currently, the software for an automated morbidity reporting system is being developed by the WRAIR Division of Preventive Medicine in conjunction with the Division of Biometrics and the Uniformed Services University of Health Sciences Department of Preventive Medicine. It is planned to utilize a mini-computer with this software in a test at Fort Campbell in late 1979.

Other individuals interested in outpatient reporting and consulted in conjunction with this project have been LTC Darlow L. Inberg, Chief, U.S. Army Patient Administration Systems and Biostatistics Activity, Fort Sam Houston, and MAJ Henry Scagliola of the Occupational and Environmental Medicine Division, U.S. Army Environmental Hygiene Agency.

20. Hepatitis: Impact on the U.S. Army and Cost/Benefit Analysis of Hepatitis B Vaccination

Although the incidence of hepatitis in the U.S. Army has decreased during this decade, it still remains as a leading cause of Non-Effective Day (NED) and accounts for about 3% of all Bed Occupancy Day (BOD). Hepatitis is a more serious problem for the Army overseas than in CONUS. During the first 6 months of 1978 hepatitis accounted for about 8% of all BOD for the Army in Europe compared to 1% in CONUS. The U.S. Army is paying a heavy toll (medical care cost and loss of manpower) for hepatitis. The present study was designed to: (1) estimate more accurately the impact of hepatitis on the U.S. Army, (2) identify the high risk group of hepatitis, and (3) analyze cost/benefit of vaccination against hepatitis B. The data source is IPDS. Cost/benefit analysis will be done when the data for efficacy of the vaccine and etiology of the hepatitis become available.

21. Demographic Variables of Tuberculosis Cases in U.S. Army

Recent experiences with tuberculosis convertors in a school age population of a U.S. Army post (Spring 1978) and reports of a similar problem in the DOD dependent school system in Europe have raised several questions in relation to the collection and use of tuberculosis data in the military system. These questions have demonstrated that the data is not being collated or interpreted to the extent possible.

Much information is being generated through the Army tuberculosis control program. This information includes data on TB screening, diagnosis and treatment of active TB and prophylaxis for convertors.

This study is being conducted to describe the population being followed through Army Medical Services for active tuberculosis. A second objective is to identify associated variables that may indicate why this group was at greater risk of developing tuberculosis. Forty-one posts in the United States including Alaska and Hawaii were selected for this study. Questionnaires were sent to the Community Health Nursing Services of each post as this service usually coordinates follow-up for tuberculosis patients. A questionnaire was requested for each person in the tuberculosis registry being followed for previously active disease. Questionnaires were sent out under USC sponsorship with a suspense date of early 1979 (April-May). The questionnaires have recently been returned and data analysis is in progress.

22. Serologic Survey of Hepatitis Antigen/Antibody Prevalence in Special Forces Troops

Little information exists concerning the exposure of Army populations to hepatitis virus types. One method of acquiring such information is to test sera remaining from previous studies. Sera from two special forces units, utilized in previous surveys and kept in storage at the WRAIR, was selected for this purpose. One group of 275 individuals participated in the Alaskan "Jack Frost" exercise of 1977 and had pre- and post-deployment sera collected for Hepatitis B Antigen/Antibody testing. No testing for hepatitis A antibody was performed at the time of sera collection due to the lack of a specific test. The second group of 106 troops participated in the 1978 "ARESH" exercise which took place in Iran. One post-deployment serum specimen was collected to check for Leishmaniasis exposure. No hepatitis testing was done in this latter group. Both groups were administered questionnaires concerning demographic variables and previous assignment information.

The present study will examine all sera for hepatitis B surface antigen, core antigen, and surface

antibody, as well as for hepatitis A antibody. Results will be correlated by computer with the questionnaire data to help elucidate which factors are related to the risk of hepatitis exposure in this specialized military population. Future studies on different Army populations will provide information to augment our knowledge of hepatitis in active duty personnel.

23. Etiology of Viral Hepatitis Requiring Hospital Admission Among U.S. Military Personnel

This is a study initiated and conducted by the Department of Virus Diseases and is being reported under Work Unit 135 "Mechanisms of Transmission of Hepatitis Viruses." The Division of Preventive Medicine is providing coding and analytic support for the project. Approximately 100 sequential active duty hepatitis patients will be entered into the study from each of five hospitals (three in Germany, one in Korea and one at Ft. Hood, TX). Two questionnaires will be completed and laboratory data from the hospitals as well as hepatitis serologic results from WRAIR will be included. The records are being transposed to computer files at the present time. Each record will consist of 5 eighty column cards. Once the file is created variables of interest will be abstracted.

Project 3M762770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 001 Epidemiologic Studies of Military Diseases

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD DRA/FAKJ036	
3 DATE PREV SUMMARY ^a	4 KIND OF SUMMARY ^a	5 SUMMARY SCTY ^a	6 WORK SECURITY ^a	7 REGRADING ^a	8A DIS'N INSTR ^a	8B DIS'N INSTR ^a	9 LEVEL OF SUM ^a
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20 RESPONSIBLE DOD ORGANIZATION				21 PERFORMING ORGANIZATION			
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish DDAN 11 U.S. Academic Institution)			
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22 GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
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				NAME:			
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24 TECHNICAL OBJECTIVE, 25 APPROACH, 26 PROGRAM (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code)							
<p>23. (U) The pathogenesis of bacterial infections of the gastrointestinal tract is being studied to establish factors and mechanisms by which disease is provoked. Through an elucidation of such elements, procedures for prevention and control of diarrheal diseases can be devised. Diarrhea is a significant problem in military personnel operating overseas.</p> <p>24. (U) The genetic control of O-antigen specificity of enteric pathogens is being studied since such cell envelope components are of importance in disease and its prevention through vaccination. Studies of enterotoxins and other virulence determinants of shigella, E. coli and Y. enterocolitica are being pursued by a genetic approach.</p> <p>25. (U) 78 10 79 09 Investigations of Y. enterocolitica have shown that their invasiveness properties are under the control of a plasmid. Contour length measurements of plasmid DNA isolated from strain Y7P has revealed two plasmid species; 42.1 megadaltons and 36.3 megadaltons. The 42 Mdal plasmid has been shown to confer magnesium oxalate sensitivity to Y. enterocolitica, a property previously shown to be related to the virulence of Y. pestis. 2 strains of Shigella dysenteriae 1 and S. flexneri 2a invade various cultured mammalian cells whether or not the cells are sensitive or resistant to Shiga cytotoxin. (For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.)</p>							

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 002 Pathogenesis of Enteric Diseases

Investigators

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Description

The pathogenesis of bacterial infections of the gastrointestinal tract, particularly those caused by Shigella, Salmonella and Escherichia coli is being studied to establish factors and mechanisms by which disease is provoked. Through an elucidation of such elements, procedures for prevention and control of diarrheal diseases can be devised.

Progress

By employing an integrated, immunologic, cytologic and genetic approach (see previous annual reports) studies in this department are concentrating on further elucidation of: (I) virulence factors and mechanisms involved in intestinal penetration and toxin elaboration by pathogens, key mechanisms by which enteric diseases are provoked; (II) the genetic control of O antigen specificity of enteric pathogens since such cell envelope components are of importance both in disease and its prevention through vaccination; and (III) the application of genetic techniques for development of live, oral vaccines against Shigellosis:

1. Investigation of Yersinia enterocolitica have shown that their invasiveness properties are under the control of a plasmid. Contour length measurements of plasmid DNA isolated from strain Y7P has revealed two plasmid species: 42.15 ± 15 megadaltons and 36.35 ± 0.89 megadaltons. The 42 Mdal plasmid has been shown to confer magnesium oxalate sensitivity to Y. enterocolitica, a property previously shown to be related to the virulence of Y. pestis.

2. Shigellae invade tissue culture cell lines which are either sensitive or resistant to Shiga toxin with equal efficiency. The plasma membrane of these cells are not measurably damaged during the process of invasion.

3. Purified rabbit ileal secretory IgA can induce antibody-dependent mononuclear cell-mediated antibacterial activity against Shigella flexneri hybrid strain X-16.

4. Using solid phase radioimmunoassay, in contrast to previous findings with hemagglutination techniques serum IgA, IgG and IgM rises could be detected in sera from volunteers vaccinated orally with the Type Ty 2a strain of Salmonella. Rises could also be detected in patients experimentally infected with Salmonella.

1. Although Yersinia enterocolitica has been recovered from sporadic human infections for over 50 years, it is only in recent years that the diseases caused by it have gained attention in the United States. Studies of the pathogenesis of acute gastroenteritis by Y. enterocolitica have now revealed that both invasive and toxigenic mechanisms are involved. Some strains of Y. enterocolitica have been shown (a) to produce a heat-stable enterotoxin (ST) similar to that of enterotoxigenic Escherichia coli and (b) to invade host tissue in a variety of animal models. The clinical signs of Y. enterocolitica infections are protean and often dependent on the age and physical health of the host. An acute enterocolitis with fever vomiting and diarrhea which occasionally is accompanied by blood and mucous, appears, however, to be the most common manifestation, particularly in infants and small children. Older children and adults have also been shown to have a terminal ileitis and mesenteric lymph-adenitis which frequently resembles an acute appendicitis. More rarely, Y. enterocolitica infections have been found to be the cause of enteric fever, septicemia, and a broad spectrum of other extra-intestinal diseases particularly in hosts that are aged, debilitated or immunologically compromised. The virulence properties and epidemiological relationships among isolates from such diverse infections and from food and water sources still remain to be characterized systematically.

Recent studies have revealed a possible plasmid control mechanism for the ability of Y. enterocolitica to cause keratoconjunctivitis in guinea pigs. In the present study of the virulence and plasmid properties of Y. enterocolitica, we likewise have found that a plasmid is associated with the invasiveness of strains. This plasmid has now been shown to be involved in the invasion and translocation of Y. enterocolitica in a murine model. Furthermore, we have revealed that strains harboring this plasmid are selectively inhibited in their growth at 37°C on magnesium oxalate agar,

a medium previously found to inhibit growth of plague bacilli which produce the V and W antigens.

The three Y. enterocolitica serotype 0:8 strains chosen for this study were kindly provided by Dr. Ira Melman, FDA, Washington DC and Dr. W.H. Lee, USDA, Beltsville, Md. Strain WA, originally isolated from a septicemic human infection, has been described previously. Strain CDC 2635, recovered from chocolate milk causing a food borne epidemic of gastroenteritis in Oneida County, NY and its isogenic derivative, TAMU-75, have also been described elsewhere. Strain Y7P was also recovered from a case of gastroenteritis.

As summarized in Table 1, these 3 strains were found to have similar invasive and toxigenic properties. Their invasiveness could readily be demonstrated in guinea pigs using the Sereney Test for keratoconjunctivitis. Unlike virulent shigellae which provoke a positive eye reaction at inocula of 1×10^9 organisms/ml, Y. enterocolitica was found to yield variable results at this dosage. Thus, to insure against the possibility of false negative reactions, broth suspensions of agar grown cells containing about 1×10^{10} organisms per ml were routinely employed for this test. When a drop of such a suspension was deposited into the conjunctival sac of a guinea pig, strains WA, CDC 2635 and Y7P repeatedly provoked positive reactions. An opaqueness usually develops in the cornea within 48 h, followed by a keratoconjunctivitis within 5 days of infection. Cultivation of the exudate from such infected eyes on MacConkey lactose agar yielded Y. enterocolitica.

These strains also were found to be lethal to mice challenged either orally or intraperitoneally (i.p.) Groups of 5 swiss albino mice (Walter Reed ICR) mice weighing 15-20 g were deprived of water for 18h and then allowed to drink ad libitum from a 50 ml water suspension of each strain containing about 1×10^9 bacteria/ml. Such oral infection of mice results in a fetal systemic disease. Within 7-14 days following infection, invasion of the liver and spleen by the challenge organism could be readily detected in moribund mice using standard bacteriological techniques. All three strains had an LD₅₀ of about 100 cells, determined from the results of i.p. injection of graded doses of the pathogens and calculated by the method of Reed and Muench.

Similar animal studies were performed with non-invasive derivatives of these strains. Strains Y7N and WA-ETBR37 were recovered following growth at 37C of the respective parental

strains in Penassay broth containing ethidium bromide ($10^{-3}M$). As shown in Table 1 these derivatives and the similarly isolated TAMU-75 strain failed to cause conjunctivitis in guinea pigs and a fatal infection in mice.

The enterotoxin properties of these strains were identical irrespective of whether they were invasive or not. No evidence of E. coli LT could be detected using the Y₁ adrenal cell assay. All strains, however, produced a heat stable enterotoxin, as shown by fluid production in the suckling mouse model. Supernatant filtrates of these strains grown in alkaline trypticase soy broth routinely yielded 80 units of toxin/ml.

The plasmid content of Y7P, WA, CDC 2635 and their respective non-invasive derivatives was next examined. A clone of Y7P, reisolated from the spleen of an orally infected mouse, was chosen for detailed study. Purified covalently closed circular plasmid DNA was recovered from Triton-X cell lysates of Y7P by means of cesium chloride-ethidium bromide density gradient centrifugation and examined for contour length under the electron microscope to determine the molecular size of plasmids. Plasmid pSC101, with a molecular weight of 6.0 ± 0.37 megadaltons (Mdals) served as the reference standard. Two plasmid classes were distinguished in Y7P; 42.15 ± 1.12 Mdals (11 molecules measured) and 36.35 ± 0.89 Mdals (30 molecules measured).

The plasmid profiles of various strains following electrophoretic analyses of ethanol precipitated DNA isolated from cleared bacterial lysates have also been compared. Such partially purified DNA was subjected to electrophoresis through 0.7% agarose gels and then examined for DNA bands by staining with ethidium bromide as previously described. The two plasmid classes of Y7P (42 and 36 Mdals), were used as references. WA and CDC 2635, both wild-type invasive strains similar to Y7P in various animal models, also possess a plasmid which is 42 Mdals in size. The non-invasive but still enterotoxigenic derivatives WA-ETBR37, TAMU-75 and Y7N, however, have lost this plasmid.

The availability of isogenic pairs of Y. enterocolitica which differ in their virulence properties have allowed studies of the nature of the invasive determinant that this plasmid confers on Y. enterocolitica. Our initial efforts have addressed properties long-recognized to be related to the virulence of Yersinia such as production of fibrinolytic

factor, coagulase and the V and W antigens of plague bacilli associated with magnesium oxalate sensitivity. Strains WA, CDC 2635, Y7P and their isogenic derivatives, however, did not possess either fibrinolytic factor or coagulase activity.

In contrast, a profound difference in growth inhibition at 37°C on magnesium oxalate agar was observed between invasive and non-invasive isogenic pairs of Y. enterocolitica. (Table 2). Single colonies of Y7P, WA, CDC 2635 and their non-invasive derivatives were grown overnight at 26° in broth and diluted in saline to a concentration of about 1×10^4 cells/ml. At this time, 10 µl samples were plated in duplicate for each of the conditions being tested. Blood agar base, lacking the other ingredients of magnesium-oxalate agar, was included as a control. It is evidence that the growth of Y7P, WA and CDC 2635 is inhibited on magnesium oxalate agar at 37° but not at 26°C. Non-invasive derivatives Y7N, WA-ETBR37 and TAMU-75, which lack the plasmid 42 mdals in size show no such growth inhibition at the elevated temperature. This growth inhibiting effect is not lethal to Y7P, WA and CDC 2635 cells. Examination of these plates under a dissecting microscope revealed a background of minute colonies which were found to develop into full size if allowed to incubate at 26°C. Preliminary characterization of Y7P, WA and CDC 2635 clones recovered at 37°C as large colonies resistant to inhibition on magnesium oxalate agar has shown them to be non-invasive and lacking the plasmid (unpublished observations).

Our finding of a selective inhibition of growth of invasive strains of Y. enterocolitica on magnesium oxalate agar thus provides a lead for establishing the nature of this plasmid determined invasive factor. Magnesium oxalate agar has been previously shown to inhibit the growth at 37°C of virulent Y. pestis which produce the V and W antigens and express a calcium deficiency affecting their ability to grow. Strains of Y. pestis which lack the V and W antigens are not inhibited in their growth at 37°C on this medium. The V protein and W lipoprotein antigens of Y. pestis produced only at 37°C, are considered to be the factors which act in combination to make plague bacilli resistant to phagocytic killing. Investigations are currently underway to determine whether invasive Y. enterocolitica produce antigens similar or identical to V and W.

Preliminary screening studies of a number of Y. enterocolitica isolates from a variety of sources also indicate that the magnesium oxalate agar inhibition test may

distinguish virulent, clinically significant isolates from other strains (unpublished observations). Such a reliable, inexpensive test would facilitate the assessment of the public health significance of Y. enterocolitica in food and water sources.

2. Bacteria of the genus Shigella produce disease by infection and destruction of the cells lining the large intestine. In this study cultured mammalian host cells were used as a simplified model of human intestinal mucosa cells. Certain strains of shigellae not only infect these cells but also produce a protein (Shiga cytotoxin) which kills mammalian cells when added exogenously. To assess the relative contribution of the above bacterial characteristics to virulence, three cell lines with differing sensitivity to Shiga cytotoxin were employed. Two cultures, Henle 407 and HeLa (CCL-2), were significantly more resistant to cytotoxin than was a third, HeLa (WRAIR). A fully toxigenic strain of Shigella dysenteriae 1 (3818T), a hypotoxigenic mutant of this strain (725), and two strains of S. flexneri 2a (24570 and M4243) were studied. Damage to the plasma membrane of infected host cells was quantitated by measuring release of (³H) uridine from labeled cells.

These studies showed that the plasma membrane of host cells was quantitated by measuring release of (³H) uridine from labeled cells.

These studies showed that the plasma membrane of host cells is not measurably damaged during the process of infection. Apparently infecting organisms are engulfed by cultured cells. In addition it was found that cytotoxin production is not directly related to infectivity in the shigella strains tested; however, the appearance of delayed cytotoxic manifestations in infected host cells was significantly accelerated when overtly toxigenic organisms infected toxin-sensitive cells. Since dysentery tends to be more severe in human infections caused by overtly toxigenic shigella strains, the above observations suggested that the clinical manifestation most directly attributable to Shiga cytotoxin may be destruction of infected colonic epithelial cells with resultant ulceration of the mucosal lining

3. We have previously demonstrated that not only IgG in human post-immunization sera, but also IgG and IgA derived from sera of patients convalescing from meningococcal disease can induce antibody-dependent mononuclear cell-mediated (ADC) antibacterial activity. We now report that rabbit ileal loop secretions lacking detectable anti-shigella IgG but containing secretory IgA (s-IgA) directed against Shigella flexneri hybrid strain X-16 (as determined by ELISA technique) can induce ADC

mononuclear cell mediated antibacterial activity. In contrast, secretions obtained prior to immunization or subsequent to immunization with normal saline were ineffective (table 3). Furthermore, by using secretions obtained on different days post-shigella X-16 immunization, we have determined that the magnitude of ADC antibacterial activity is directly proportional to the amount of anti-shigella X-16 s-IgA present (table 4). Since mononuclear cells present in mucosal tissues have been shown to be capable of mediating antibody-dependent cellular cytotoxicity, it is possible that this newly discovered antibacterial role of s-IgA is an important local host immune defense mechanism which functions by limiting bacterial multiplication in secretory tissues and thereby decreases the opportunity for systemic invasion by pathogenic bacteria. IgA may be uniquely suited to this tissue-associated role since it does not activate the complement cascade and, therefore, does not, in this ADC antibacterial function, concomitantly generate local immunopathology and inflammation. Demonstration of s-IgA dependent mononuclear cell mediated antibacterial activity may be significant in evaluating the ability of potential anti-shigella vaccines to induce a local immune response which is effectively antibacterial.

4. The UDP - Glucose-4 - Epimeraseless mutant of *Salmonella typhi* (Ty 21a) is currently being evaluated as a live oral vaccine against typhoid fever (1). In the published data only 17% of the volunteers vaccinated orally had a 4-fold rise in 0 antibody, yet only 7% of vaccines developed typhoid when experimentally challenged, as compared to 53% of non-vaccinated controls (1). This would suggest that serum antibody did not correlate and was not involved in immunity. These assays were performed using standard hemagglutination techniques and because these techniques primarily measure IgM it was felt that these sera should be re-examined using the technique of class specific solid phase radioimmunoassay. Eight pairs of sera consisting of a pre bleed and post oral vaccination sera were tested for IgG, IgA and IgM levels against lipopolysaccharide (LPS) from the Gal E vaccine grown in the presence of galactose or the presence of glucose, LPS from wild type *Salmonella typhimurium*, LPS from *Shigella*, and LPS from the LPS core defective mutants J-5 and Re. No significant rises in antibody were found in the volunteers for any class of antibody against LPS from either *Shigella*, the J-5 or Re mutants. The data for class specific rises against galactose grown Ty 2a and wild type LPS antigens is shown in Table 5. The solid phase detected a rise in 6/8 vaccinated volunteers of IgA antibody against LPS from Ty 2a antigen, 5/8 in the IgG class and 6/8 in the IgM class. Rises against wild type LPS were not as high or as consistent. The

rises in IgA class were as high as those in the IgG class which may be related to the fact that this is an oral vaccine. Only 2 of 8 gave rises against wild type antigen of the IgM class. This is consistent with the HA rises against wild type O antigen previously reported. These data explains their results and demonstrates that significant serum rises do occur following oral vaccination when the vaccine antigen is used for measuring antibody.

Thirty eight sera from 6 volunteers experimentally infected with *Salmonella* were tested for sequential rises class specific against LPS from wild type *Salmonella*. Serum from four of the six volunteers demonstrated consistent rises in all three classes. Thirty six sera from patients experimentally infected with *Shigella flexneri* were examined for rises in antibody against LPS from the infecting strain. None had IgM rises, 4/6 had IgA rises and 3/6 had IgG rises. Rises in IgA and IgG classes of antibody can therefore be measured in the sera of most but not all volunteers either vaccinated orally or experimentally infected with salmonella or shigellae.

Table 1
Virulence properties of Y. enterocolitica strains

Virulence Properties									
Murine Challenge*									
Strain	Serenity test	Guinea Pig	LD ₅₀ (i.p.) # bacteria	Oral Infection**			LT	ST	
				Death	Liver***	Spleen***			
Y7P	+		1.4 x 10 ²	25/25	+	+	-	+	
Y7N	-		10 ⁸	0/25	nt	nt	-	+	
WA	+		1.1 x 10 ²	25/25	+	+	-	+	
WA-ETBR37	-		> 10 ⁸	0/25	nt	nt	-	+	
CDC 2635	+		9.3 x 10 ¹	25/25	+	+	-	+	
TAMU-75	-		> 10 ⁸	0/25	nt	nt	-	+	

* The experiments were terminated 21 days after infection.
 ** Mice were allowed to drink ad libitum from a 10⁹/ml suspension of cells.
 *** Recovery of challenge strains on Mac Lactose agar from moribund animals.
 **** nt, not tested.

Table 2

Growth of invasive and non-invasive isogenic pairs of Y
enterocolitica on magnesium oxalate agar **

Strain	Average number of colonies*			
	Magnesium oxalate agar*		Blood agar base	
	26C	37C	26C	37C
Y7P	154	1	163	147
Y7N	183	181	176	175
CDC 2635	196	< 1	206	193
TAMU-75	165	153	162	169
WA	140	< 1	147	148
WA-ETBR37	161	165	162	154

* 10 μ l samples were spread in duplicate for each condition.

** Ingredients per liter: Blood agar base, 40 g; $MgCl_2$ (0.25 M), 80 ml; sodium oxalate (0.25 M), 80 ml; glucose (1M) 10 ml; distilled water, 830 ml.

Table 3

The ability of rabbit ileal secretions containing anti-shigella X-16 secretory IgA to induce antibody dependent mononuclear cell-mediated antibacterial activity against shigella X16

Serum	Dilution	No. of viable bacteria/ml $\times 10^{-3}$	Antibacterial activity (%)
None	-	> 11.1	0*
Non-immune	Neat	10.8	12*
	1/2	10.7	14*
	1/4	> 11.1	0*
	1/8	> 11.1	0*
Immune	Neat	1.7	85
	1/2	3.9	74
	1/4	4.2	63
	1/8	6.8	39
	1/16	7.7	29
	1/32	8.5	22*
	1/64	9.2	18*

* Antibacterial activity not significant.

Table 4

The relationship between the amount of anti-shigella X-16 secretory IgA and its ability to induce antibody-dependent mononuclear cell-mediated antibacterial activity

Day post-immunization that secretions are collected	Units anti-shigella X-16 secretory IgA* in collected secretion	Antibacterial activity**
1	.063	0
4	.136	35
7	.234	63
11	.328	92
21	.966	97

* As measured in ELISA assay.

**All values represent activity at 1/16 dilution.

Table 5

Class specific antibody rises in volunteers orally vaccinated with
Ty 21a Salmonella typhi vaccine

Volunteer Pair	IgA *		IgG *		IgM *	
	Ty 2a LPS	Wild LPS	Ty 2a LPS	Wild LPS	Ty 2a LPS	Wild LPS
1 pre	.05	0.13	2.51	1.27	1.96	5.57
post	7.58	2.22	8.01	9.82	9.51	7.76
2 pre	0.07	0.17	0.06	0.28	3.0	4.33
post	0.27	0.17	0.07	0.28	8.17	8.08
3 pre	0.03	0.14	1.42	0.38	0.31	2.49
post	6.94	0.25	8.25	0.56	16.7	6.5
4 pre	0.01	0.1	0.1	0.17	0.77	1.42
post	0.05	0.13	0.22	0.17	4.26	2.07
5 pre	0.02	0.09	0.08	0.53	0.6	0.76
post	.51	0.64	10.72	2.65	9.3	4.53
6 pre	0.04	0.19	0.06	0.23	2.6	2.9
post	4.17	6.39	0.10	2.22	21.8	18.6
7 pre	0.8	1.22	0.26	3.21	2.3	3.8
post	8.06	6.42	2.09	4.35	3.8	4.5
8 pre	0.53	0.36	0.45	1.19	2.5	4.6
post	8.02	0.34	2.13	0.63	2.1	5.3
Mean pre	0.19	0.3	0.68	0.89	1.67	3.2
Mean post	4.56	2.07	4.02	2.57	9.45	7.1

* All antibody levels in micrograms/ml.

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 002 Pathogenesis of Enteric Diseases

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION*	2 DATE OF SUMMARY*	REPORT CONTROL SYMBOL DD FORM 1498 (AR) 636	
3 DATE PREVIOUS SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY*	6 WORK SECURITY*	7 REGRADING*	8A DISSEM INSTR*	8B SPECIFIC DATA CONTRACTOR ACCESS	9 LEVEL OF SUM A. WORK UNIT
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10 NO / CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62770A	3M762770A802	00	003			
b. CONTRIBUTING	XXXXXXXXXX	Cards 114F					
11 TITLE (Provide with Security Classification Code)*							
(U) Histopathologic Manifestations of Military Diseases and Injuries							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS*							
002600 Biology							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
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17 CONTRACT/GRANT				18 RESOURCES EST. TYPE		19 PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE				PRECEDENT*			
b. NUMBER*				FISCAL YEAR		20 FUNDS (in thousands)	
c. TYPE NA				79		5 499	
d. KIND OF AWARD				80		5 429	
19 RESPONSIBLE DOD ORGANIZATION				20 PERFORMING ORGANIZATION			
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11 GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Hase, T., Tseng, J., Henley, G			
				NAME: Cho, H.			
21 KEYWORDS (Provide each with Security Classification Code)							
(U) Immune responses; (U) Intestine; (U) Electron microscopy; (U) Dysentery							
22 TECHNICAL OBJECTIVE, 23 APPROACH, 24 PROGRESS (Provide individual paragraphs identified by number. Provide rest of each with Security Classification Code)							
<p>23(U) To define histopathologic manifestations of injuries and diseases which have current or potential problems in military personnel. The current effort is directed toward studies of enteric diseases and immunologic responses to enteric and other infections. These studies provide a basis for a comprehension of pathogenesis, therapy, and determination of prognosis in infectious diseases of military personnel.</p> <p>24(U) Various morphologic techniques including histology, histo- and cytochemistry, autoradiography, immunofluorescent microscopy, transmission and scanning electron microscopy are employed. Various immunologic techniques have also been utilized.</p> <p>25(U) 78 10-79 09 Light and electron microscopical studies of unculturable gastric spirochetes in clinically normal rhesus monkeys have defined their incidence, ultrastructural features and host cell relationship. Sequential studies of the mouse lung model of Neisseria gonorrhoeae infection have characterized the host/bacteria relationship and the phagocytosis and destruction of the bacteria in lymphocytes and macrophages. The cellular kinetics of experimental intraperitoneally induced scrub typhus infections are in progress. Studies on the cellular immune system of the gastrointestinal mucosa and its associated immune system have been initiated. The ultrastructural study of blood forms of Trypanosoma brucei have been completed. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.</p>							

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 003 Histopathologic Manifestations of Military
Diseases and Injuries

Investigators:

Principal: Akio Takeuchi, M.D.

Associates: Han Y. Cho, Ph.D., Tatsuo Hase, M.D.,
SFC Garnett Henley, M.S., Jeenan Tseng, Ph.D.

Description

To define histopathologic manifestations of injuries experimentally produced and diseases which present current or potential problems in military personnel. The current effort is directed toward studies of diseases of the digestive tract and immune responses due to infection. These studies provide a basis for a comprehension of pathogenesis, scientific treatment, and determination of prognosis in diseases and injuries in military personnel. A multi-disciplinary approach including conventional histology, histo- and cytochemistry, autoradiography, radio-tracer methods, various immunological techniques, immunofluorescent microscopy, transmission and scanning electron microscopy is employed.

Problem and Progress

This work unit consists of studies of histologic manifestations of acute diarrheal diseases of infectious origin and collaborative studies of experimental gonococcal, rickettsial and trypanosomal infections with other departments of the WRAIR. Recently we have initiated studies on the immune system of the digestive tract.

I. Studies on Spirilla in the Stomach of the Rhesus Monkey

The presence of spirilla in the gastric mucosa has long been recognized in various animal species including monkey and man. Yet, little is known about their relationship to the host and their presence is often overlooked. These spirilla have never been cultured on an artificial medium, and therefore, nothing is known about their physico-chemical characteristics. At present, these spiral-shaped organisms are referred to as gastric spirilla (GS) because of their characteristic shape as revealed by light microscopy.

Results

We have found spirilla in the stomach in each of the 45 monkeys examined. In paraffin sections GS are best demonstrated by silver stains. When stained by hematoxylin-eosin (HE), GS may be mistaken for strands of mucus. In thick sections of Epon-embedded tissue, GS are recognized as "corkscrews" with up to 12 coils. They are 8 μ m long and 0.7 μ m wide and have characteristic bipolar flagella. They are concentrated in the gastric glands at the isthmus and are less frequent at the neck and base, while they are absent in the gastric lumen. GS are closely associated with parietal cells and are capable of penetrating their cytoplasm. GS elicit neither changes of host cyto-components nor an inflammatory response in the gastric mucosa.

II. Experimental Neisseria gonorrhoeae Infection

Gonorrhea is one of the most common venereal diseases in both civilian and military personnel. Yet, we have little knowledge concerning pathogenesis and immune response of this important sexually transmitted infection

To date, numerous investigators have tried to produce experimental gonococcal infections in various animals by different modes of infection with no success. Recently, we have established experimental gonococcus infection in adult mice and guinea pigs by intranasal administration of freshly cultured Neisseria gonorrhoeae obtained from human patients.

Results

By intranasal challenge, mice were infected with 10^8 Neisseria gonorrhoeae organisms of a primary isolation culture of colony type 1 gonococci (GC) and sacrificed at 0, 3, 6, 12, 24, and 48 hr. after infection. One-half lung was examined histologically and the other half for GC organisms. 2×10^6 to 2×10^7 GC were recovered from mice sacrificed 0, 3, and 6 hr. At 12 hr GC were detected in small numbers or not at all and could not be cultured thereafter.

Histologically, at 0 and 3 hr GC were free in the bronchial lumen, attached to epithelial surfaces of bronchi and to alveolar septa. Migration of leukocytes (LC) was minimal and no intracellular GC were identified. At 6 hr. the number of LC was markedly increased while that of macrophages was minimal. Phagocytosed GC

were found mainly in LC which filled the bronchial lumen. Extracellular GC were still attached to alveolar cells. At 12 hr. the number of GC containing LC and macrophages were further increased, however, extracellular GC were absent. At 24 hr. both LC and macrophages were present in bronchi and alveoli but neither showed phagocytosed GC. At 48 hr. cellular infiltrates were minimal or totally absent.

We are studying on the interaction between GC and the bronchial epithelial cells, LC and macrophages by transmission and scanning electron microscopy.

III. Studies on Experimental Scrub Typhus Infection

Transmission electron microscope studies on cells of the peritoneal exudate following intraperitoneal inoculation of mice with the La (*Leptotrombidium arenicola*) strain of scrub typhus rickettsia are in progress.

IV. Studies on Trypanosoma brucei Infections

Utilizing 3,3'-diaminobenzidine, a cytochemical proton donor, electron microscope observations have clarified the morphology of the kinetoplast-mitochondrial network in Trypanosoma brucei blood forms.

V. Studies on the Immune System of the Digestive Tract

With a newly assigned immunochemist, we have initiated a fundamental study on the immune system of the gastrointestinal tract. Currently our efforts are directed toward the characterization of IgA precursor cells which are responsible for humoral immunity in the intestine.

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 003 Histopathologic Manifestations of Military
Diseases and Injuries

Presentations

1. Takeuchi, A.: Pathology of bacillary dysentery in the rhesus monkey. NIH Symposium on diseases of the digestive tract of the subhuman primate, March 3, 1979.
2. Schneider, H., Bernard, E. and Takeuchi, A.: Responses of mice to intranasal challenge with Neisseria gonorrhoeae. Annual Meeting of American Society for Microbiology, May 7, 1979.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL DD FORM 1 (7/8)636	
3. DATE PREV. SUMRY 78 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY* U	6. WORK SECURITY* U	7. REGRADING* NA	8. DISSEM INSTR* NL	9. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT
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12. SCIENTIFIC AND TECHNOLOGICAL AREAS* 0100100 Microbiology 008800 Life Support 002600 Biology							
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19. RESPONSIBLE DOD ORGANIZATION			20. PERFORMING ORGANIZATION				
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21. GENERAL USE			SOCIAL SECURITY ACCOUNT NUMBER:				
Foreign intelligence not considered			ASSOCIATE INVESTIGATORS				
			NAME: CHIENEY, CPT, C.P.				
			NAME: DUNN, MAJ, MA.				
22. KEYWORDS (Provide EACH with Security Classification Code) (U) Diarrhea (U) Absorption (U) Immunology (U) Myoelectric Activity (U) Bacterial Mucosal Adherence (U) Liver Fibrosis (U) Schistosomiasis (U) E.Coli							
23. TECHNICAL OBJECTIVE* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Provide text of each with Security Classification Code.) (U) Research efforts in this department continue to be directed toward Gastrointestinal diseases of military importance. Focus is on enteropathogenic bacterial diarrheal disease including pathogenic E.Coli, Salmonellosis and Shigellosis. These have critical military relevance because of their influence on troop mobility. Studies are also performed on the determinants of fibrosis in parasitic liver disease (schistosomiasis).							
24. (U) Studies of bacterial diarrhea are being conducted in 4 general areas 1) Intestinal cell membranes as determinants of bacterial colonization. 2) Cellular immune response to intestinal infection. 3) Pharmacologic modification of effects of infections on intestinal transport and 4) motility. Studies utilized in vivo intestinal perfusions of rabbits and rats, rat ileal loop models, Ussing chambers, in vivo recording of intestinal myoelectric activity and in vitro agglutination of intestinal membrane fractions. Isolation and functional characterization of intestinal lymphocytes is performed.							
25. (U) 78 10 - 79 09, Cell Membranes - the optimum conditions for bacterial adherence have been defined and the bacterial surface structures mediating adherence have been identified. Immunology - intestinal lymphocytes are isolated from animals with enteric infections their function determined and a suppressor effect on these lymphocytes documented. Liver Fibrosis - rate limiting steps in hepatic fibrosis and their pharmacologic modification are studied. Transport and motility - mechanisms by which methylprednisone and other drugs increase mucosal water and electrolyte absorption are studied in vitro. The effect of pressure and distention on intestinal secretion and the response of intestinal myoelectric activity to enteric infection is studied. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sept 79.							

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 005 Gastrointestinal Diseases of Military
Importance

Investigators

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Description

The research activities in this Department have continued to focus on:

I Diarrheal diseases caused by the toxigenic and/or invasive enteropathogenic E.Coli, Salmonella, Shigella and, to a lesser extent, Cholera. Four basic sets of questions are being asked about the pathogenesis of these diseases.

A. Role of Mucosal Adherence in Bacterial Colonization of the Intestine - How do disease causing bacteria interact with the surface of the gastrointestinal tract? What characteristics of the intestinal surface permit disease causing bacteria to adhere to and colonize the small intestine? What characteristics of the bacterial surface enable organisms to adhere to and colonize the mucosal surface? What characteristics of the intestinal surface permit specific interactions with toxins produced by bacteria? Can specific interactions of bacteria or their toxins be altered by orally administered agents?

B. Role of Host Immune Mechanisms - How do disease causing bacteria interact with the normal cellular (lymphocyte, macrophage, polymorphonuclear leukocyte) and humoral (antibody) immune defense mechanisms in the intestine? What are the most effective means of inducing active cellular and humoral immunity to enteric infection (i.e., how can an effective vaccine for enteric infection be produced? What factors (antibody, complement) enable lymphocytes to kill enteric bacteria in vitro? Are these factors of equal importance in an in vivo model of enteric infection?

C. Alterations of Intestinal Transport - What enzymatic and hormonal mechanisms mediate bacterial-induced secretion of water and electrolytes by the intestine? What are the normal mechanisms for salt and water absorption? How do absorptive and secretory mechanisms interact? Do they share any pathways? Can pharmacologic agents increase salt and water absorption in the face of infection or decrease secretion induced by bacterial toxins?

D. Alterations in Intestinal Motility - What are the mechanisms by which bacterial toxins change small bowel myoelectric patterns? Do toxins injected into the bowel lumen change myoelectric patterns and motility in the same fashion as the native infection? How soon after clinical disease can antibiotics reverse abnormal myoelectric patterns and abnormal motility in the intestine?

II Studies on liver fibrosis in schistosomiasis, which is the most prevalent form of serious liver disease world-wide, are being continued. Liver fibrosis is that process that determines reversibility or progression to chronic illness in all liver disease and the key biochemical event in this process is the deposition of collagen. Studies in this Department have focused on collagen metabolism in liver tissue from two animal models of schistosomiasis as well as from patients with this disease.

I Bacterial Diarrhea

A. Role of Mucosal Adherence in Bacterial Colonization of the Intestine.

Two assays were developed to quantitate the adherence of an E.coli strain (RDEC-1) known to colonize the mucosal surface of the small intestine of rabbits to brush borders isolated from rabbit intestinal epithelial cells. In the first assay, the mean adherence per rabbit brush border (RBB) was determined by counting the number of organisms adhering to each of 40 brush borders under phase microscopy. The mean adherence of RDEC-1 (11.5 ± 0.7 per RBB) was significantly greater than adherence of 2 nonpathogenic strains: HS (2.7 ± 0.4 per RBB) and 640 (0.8 ± 0.1 per RBB). A similar distinction between the adherence of RDEC and the control (nonadherent) organisms could be made more rapidly by determining the percentage of the total number of brush borders which had 10 or more adherent organisms and this second assay was used to define the optimum conditions for adherence. Maximum adherence was seen within 15 minutes. Adherence was temperature dependent with adherence after 1

minute at 37°C being 4 fold greater than that at 4°C. The pH optimum for adherence was between 6.5 - 7.0 and adherence was abolished below pH 5.0. Using the first, more sensitive assay, the effect of electrolytes, and a number of hexoses and hexosamines, on adherence was analyzed. RDEC-1 adherence was inhibited at high ionic strengths however, adherence was not influenced at moderately high concentrations (20mg/ml) by either D-mannose or L-fucose, in contrast to the case for other reported enteric pathogens. These two quantitative in vitro assays for adherence produce consistent results and have been used to partially characterize the adherence of RDEC-1 to RBB. (11)

Pili have been implicated as the factors involved in adherence of bacteria to mucosal surfaces. To determine whether pili are the structures responsible for adherence of an enteropathogenic E.coli, RDEC, to rabbit intestinal brush border membranes (RBB), we transferred the adherence properties to another organism or suppressed their phenotypic expression and looked for the presence of pili. Genetic transfer of RDEC adherence properties to a non-piliated (P-), nonadherent (A-) Shigella strain was accomplished. The resulting adherent (A+) Shigella strain was demonstrated to be piliated (P+) by electron microscopy (EM). In other studies, the phenotypic expression of adherence was suppressed by growth of RDEC in Brain Heart Infusion medium. The phenotypically A- organisms were shown to be P- by EM. SDS acrylamide gel electrophoresis of whole organisms revealed that P+A+ organisms contained two proteins (MW 44,000 and 42,00) not expressed on P-A- organisms. Hydrophobic interaction chromatography showed adsorption to phenyl sepharose of 90% of P+A+ organisms and of only 13% of P-A- phenotypes. These studies demonstrate that the expression of RDEC pili correlates with adherence of these organisms to RBB and suggests a hydrophobic interaction may be involved in the adherence of structures with 44,000-42,000 MW subunits to the mucosa. (1,12)

Adherence of bacteria to the intestinal epithelium can be mediated by lectin-like interactions with cell surface saccharides. However, other mechanisms, including hydrophobic interactions with the cell membrane, may be involved. We studied the hydrophobic surface properties of related adherent and nonadherent organisms using hydrophobic interaction chromatography. We compared the retention of these organisms on a column of phenyl-substituted sepharose with their adherence to rabbit BBM and with the presence of surface pili as seen by electron microscopy (EM). The organisms tested were: RDEC, a mannose resistant adherent E.coli producing diarrhea in rabbits

with ileal colonization; D-1-5, an adherent product of the mating of RDEC and a shigella; D-1-2, a nonadherent product of the same mating; and 640, a nonadherent E.coli from normal rabbits. RDEC and D-1-5 were also tested after the phenotypic expression of adherence had been suppressed by growth in Brain-Heart Infusion (BHI) medium. Degree of hydrophobic interaction was expressed as the % retention of 10^8 organisms on a 0.8ml column of phenyl-sepharose at pH 7. Results are tabulated:

Organism	Growth Medium	Adherence to BBM	Pili (EM)	% Retention on Phenyl Sepharose (+/- SE)
RDEC	Std.	+	+	87.6 (+/- 1.2)
D-1-5	Std.	+	+	89.5 (+/- 10.6)
RDEC	BHI	-	-	4.3 (+/- 4.3)
D-1-5	BHI	-	-	15.0 (+/- 1.7)
D-1-2	Std.	-	-	26.6 (+/- 14.9)
640	Std.	-	+	35.2 (+/- 3.5)

In summary 1) the adherent pathogen RDEC has hydrophobic surface properties and pili 2) transfer of RDEC adherence factors to D-1-5 correlates with transfer of hydrophobic properties and pili 3) suppression of adherence in RDEC and D-1-5 results in loss of both hydrophobicity and pili 4) nonadherent 640 is mildly hydrophobic but has pili distinct from those of RDEC. We conclude that adherence of RDEC to rabbit BBM correlates with hydrophobic surface properties which may be conferred by the presence of specific pili. (2,13)

A major determinant of the pathogenicity of Escherichia coli (E.coli) is the ability to colonize the mucosal surfaces of the host bowel. Recently, an enteropathogenic E.coli (RDEC) isolated from young rabbits with diarrhea has been demonstrated to adhere to and agglutinate brush borders (BB) from normal rabbit ileal epithelial cells. To determine whether this in vitro adherence correlated with in vivo infectivity, we quantitated RDEC adherence to brush borders from rabbits, rats and guinea pigs, then compared these results with the ability of RDEC to colonize and elicit disease in these animals. In the first study, specificity of in vitro RDEC adherence to BB isolated from ileal epithelial cells of rats, guinea pigs and rabbits was examined. BB (10^5) were incubated with 10^8 organisms for 15 min at 23°C, examined under phase microscopy and adherence was reported as the number of adherent RDEC per BB. Adherence of RDEC to rabbit BB (11.5 ± 1.2 RDEC/BB) was significantly greater ($P < .001$) than the adherence of RDEC to rat or guinea pig BBs, 0.9 ± 0.4 and 0.05 ± 0.01 . In the second study, rats guinea pigs and rabbits were orally

inoculated with either RDEC or sterile culture broth. Animals were examined daily and sacrificed at the onset of diarrhea or after 10 days. One cm segments of jejunum, ileum and caecum were aseptically removed and E.coli colony forming units (CFU)/g tissue determined. Representative E.coli colonies were serotyped to document the presence of RDEC. All RDEC inoculated rabbits developed diarrhea and displayed heavy colonization in the ileum and caecum with a mean of 4.3×10^8 and 1.7×10^8 CFU/g tissue. In contrast, RDEC inoculated rats and guinea pigs failed to develop diarrhea and RDEC failed to colonize any segment of bowel in either of these animals. In summary, 1) in vitro RDEC adherence to isolated BB correlates with in vivo colonization and infectivity 2) these properties are species specific and 3) presence of specific host receptors for bacteria appears to be a determinant of species susceptibility to enteric colonization and infectivity. (3,14)

B. Role of Host Immune Mechanisms.

Interaction between gut associated lymphoid tissue (GALT) and systemic lymphocytes may be important in the local regulation of the intensity of the host immune response to foreign antigens. We investigated the ability of the rabbit ileal lamina propria (LP) mononuclear cells (lymphocytes and macrophages) isolated by collagenase digestion to influence the autologous splenic lymphocyte response to PHA. Thymidine ³H uptake by 3×10^5 splenic lymphocytes in replicate microcultures was inhibited approximately 25, 50 and 75% by the addition of 0.3 , 0.75 and 1.5×10^5 LP cells respectively in repetitive experiments. The suppression cannot be attributed to PHA binding by the LP cells since experiments which maintained the total cell population constant (by reducing the splenic cells 5, 10 and 25% and adding back equal numbers of LP cells) also produced inhibition at 15, 20 and 90% respectively in repetitive experiments. The suppression is blocked 50-100% by irradiating the LP cells with the macrophage sparing dose of 6,000 R. This suggests that the suppression is an effect of the LP lymphocytes. The above experiments were performed in normal adult New Zealand white rabbits. A similar suppression was seen using LP cells isolated from animals which had been hyper-immunized with foreign proteins via a chronic ileal (Thiry-Vella) loop. However, suppression of splenic lymphocytes was not seen using LP cells isolated from germ deprived animals which had been reassociated with a defined, but limited, enteric flora. These latter observations suggest a relationship of the suppressor effect to the degree of intestinal immunization. Suppression was not seen if Peyer's patch or mesenteric lymph node cells replaced the LP cells. These data demonstrate

suppression of the splenic lymphocyte PHA response by the LP mononuclear cells and suggest that the LP cells might prevent the systemic T lymphocytes from participating in an immune mediated inflammatory response in the intestine of the normal rabbit. (15,16)

The functional capacity of intestinal lymphocytes from patients with inflammatory bowel disease (IBD) was examined. Cytotoxic lymphocytes may mediate (IBD) but have not been demonstrated in normal or IBD intestinal mucosa. Therefore we have examined human intestinal mucosa for the presence of cytotoxic lymphocytes. Using a sequential dithiothreitol-EDTA-collagenase technique, we examined lymphocytes obtained from mucosa of 18 normal (uninvolved tissue from colon cancer patients) human colons and 14 colons involved with IBD, all obtained at surgery. Antibody dependent cellular cytotoxicity (ADCC) spontaneous cell mediated cellular cytotoxicity (SCMC) and lectin--induced cellular cytotoxicity (LICC) were examined. The percentage of specimens with cytotoxic cells was as follows:

Assay	Targets	Normal		IBD	
		Unsep	moDepl	Unsep	moDepl
ADCC	Chick RBC	83%	83%	100%	100%
	Chang Line	20%	20%	30%	33%
SCMC	K562 Line	38%	33%	17%	0%
	Chang Line	31%	20%	25%	0%
LICC	Human RBC	81%	100%	91%	67%

ADCC and LICC activity persisted but SCMC decreased following G-10 macrophage depletion. No striking difference in cytotoxic capability was noted between normal and IBD intestinal cells. Surface characteristics revealed 35.5 ± 5.2 (mean \pm S.E.M.) E rosetting, 6.6 ± 1.6 EA rosetting, 13.8 ± 2.8 SIg positive, and 10.8 ± 1.6 esterase positive cells. No difference in surface characteristics between unseparated IBD and normal intestinal mucosal mononuclear cells was noted. These experiments demonstrate: 1) Fc-receptor bearing lymphocytes as well as T and B cells and macrophages are present in normal and IBD human intestinal mucosa. 2) Lymphocytes from both normal and IBD human intestinal mucosa mediate ADCC and LICC with red cells as targets. 3) Intestinal mononuclear cells occasionally mediated ADCC and SCMC with cell lines as targets. 4) No major difference in cytotoxic capabilities or surface characteristics between normal and IBD intestinal lymphocytes has been observed. (17)

The following study on antibacterial activity of human peripheral blood mononuclear cells was performed in collaborat-

ion with DCD&I. In cooperation with human heat-inactivated antisera from adults immunized with group C meningococcal polysaccharide, normal human peripheral blood mononuclear cells significantly decreased the viability of group C meningococci (Mgc) in vitro. K lymphocytes (Null cells) and monocytes, (but not T or B lymphocytes) were capable of effecting antibody-dependent cell-mediated (ADC) antibacterial activity in this system. The degree to which meningococcal viability was decreased was function of the length of the test incubation, the concentration of effector cells, and the amount of antiserum used in the assay. When specific antibodies directed against Mgc were adsorbed from the antiserum, cell-mediated antibacterial activity was abolished. ADC antibacterial activity was also abrogated by performing the assay at 4°C or by heating effector cells to 46°C for 15 min before the assay. A clinical study of cellular immune function of patients taking the H₂ receptor antagonist cimetidine was performed. The data suggest that K cells (as well as monocytes) may play a role in host immune defense against pathogenic bacteria. (18)

It has been proposed that cimetidine may adversely affect cellular immune function in patients being treated for duodenal ulcer disease. We therefore studied nine patients with duodenal ulcer disease immediately prior to, after 2-3 weeks and after 6-8 weeks of cimetidine treatment. There were thirteen normal controls not taking cimetidine. Whole blood cimetidine and serum immunoglobulin levels (IgG, IgA, and IgM) were determined. Peripheral blood lymphocytes obtained by ficoll hypaque separation were analyzed as to their surface characteristics, cytotoxic capabilities, and responsiveness to mitogens. Cytotoxic and mitogenic assays were performed in paired media, one containing the subjects's own serum drawn simultaneously with the cells and the other containing fetal calf serum. Therapeutic blood levels of cimetidine were present in all patients at the time of assays. Total white blood cell and mononuclear cell counts did not change in the interval studied. Although patient's granulocyte counts, which were moderately elevated before cimetidine therapy, fell to within the normal range by 6-8 weeks of therapy, no patient became granulocytopenic. Surface characteristics (percent cells esterase positive; surface immunoglobulin positive; E rosette; EA rosette; and EAC rosette forming) did not change during the treatment period. The ability of peripheral blood cells to mediate spontaneous cell mediated cytotoxicity of K-562 cells was markedly impaired in duodenal ulcer patients prior to treatment and returned to normal after 2-3 weeks of treatment. The capacity of patient's peripheral blood cells to mediate antibody dependent cellular cytotoxicity to either chick RBC or

K-562 cell line cells, or lectin induced cellular cytotoxicity to human RBC was not altered by cimetidine during the course of therapy. Likewise, mitogenic responsiveness to PHA, Con A, and PWM remained unchanged in patients while treatment with cimetidine was being given. Furthermore, there was no difference in any of the results between assay run in patients own serum from that day in comparison to fetal calf serum. Finally, serum immunoglobulin levels did not change during cimetidine treatment. We conclude that cimetidine therapy in vivo for up to eight weeks induces no marked enhancement or alteration of general cellular immune function. (19)

The cellular immune status of patients with Whipple's disease, a chronic intracellular infection of the gastrointestinal tract with "bacillary bodies" was investigated. No consistent functional immunologic defect has been found in our studies of the mitogenic responses and cytotoxic abilities of peripheral blood lymphocytes from three patients with Whipple's disease. One patient was studied both during active disease and after therapy, whereas the other two were studied only after therapy. All three patients had a decreased percentage of peripheral blood T lymphocytes; however, their mitogenic responses to phytohemagglutinin, concanavalin A and pokeweed mitogen were usually as vigorous as those of concurrently examined normal subjects. In addition, all three patients were able to produce cutaneous hypersensitivity responses. Likewise, cells from these patients usually produced antibody-dependent cell-mediated cytotoxicity at control levels, but spontaneous cell-mediated cytotoxicity was decreased in two convalescent patients. No serum inhibitor could be demonstrated in those instances where patient mitogenic or cytotoxic responses were lower than those of controls. The present study indicates that if an immunologic defect exists in patients with Whipple's disease, its expression is not as broad or clearly delineated as earlier studies have suggested. More detailed longitudinal studies of immunologic functions in patients with Whipple's disease may help to clarify the nature of immunologic responses in these individuals. (20)

C. Alterations of Intestinal Transport.

The normal mechanisms of electrolyte transport in the rat ileum were defined. The short-circuit current (Isc), potential difference (PD), tissue conductance (Gt), and Na and Cl fluxes in the short-circuit state across rat ileum were studied in Ussing chambers using a variety of bathing solutions. In Ringer's solution, Isc exceeded net Na absorption and net Cl secretion occurred. Addition of 10 mM glucose

were present in the rat colon 6 or 72 hours after methylprednisolone treatment. The increase in ileal guanylate cyclase activity and cGMP concentration coincided with increases in ileal short circuit current (Isc), potential difference (PD), net Cl secretion, and serosal to mucosal unidirectional Cl flux. In contrast, in the colon, where no changes in guanylate cyclase activity were observed, methylprednisolone did not alter electrolyte transport in vitro. The methylprednisolone-induced increase in ileal guanylate cyclase activity was not prevented by pretreatment with the mineralocorticoid competitive inhibitor spironolactone. In addition, the mineralocorticoid deoxycorticosterone acetate (0.15mg/100g) did not affect ileal or colonic mucosal guanylate cyclase activity. These results suggest that the changes in chloride transport which occurred in the small intestine 6 hours after methylprednisolone administration may be due to alterations in guanylate cyclase activity, and further support the role of cGMP as an intracellular mediator of ileal chloride secretion. (22)

The mechanism of action of the intestinal secretagogue serotonin (5HT) was investigated in the following studies: The current evaluation of the mechanism by which serotonin induces ileal secretion in vivo has revealed that serotonin effects active Na and Cl transport, specifically decreasing neutral NaCl absorption. Previously many studies have demonstrated that serotonin increases ileal motor function. Of interest is that the serotonin effect on active electrolyte transport occurred only from the serosal surface and was dependent on the presence of serotonin either on the serosal surface or in the blood. This is different than the previously described effect of serotonin on intestinal motor function, which occurred preferentially from the mucosal surface. In addition, serotonin did not appear to effect intestinal electrolyte transport through a) interaction with the adenylate cyclase-cAMP system; b) alteration of ileal Na-K-ATPase activity; c) alteration of glucose or amino acid absorption or glucose-dependent water and Na absorption; d) changes in ileal surface area; e) alteration of the ileal intercellular space and probably of serosal hydrostatic pressure; f) alteration of ileal blood flow; or g) changes in ileal permeability. (23)

We previously have demonstrated that 5HT causes water and electrolyte secretion in rabbit ileum in vivo. These studies were performed to determine the mechanism of that secretion. The effect of 5HT on active electrolyte transport was determined in vitro on a stripped preparation of histologically normal rabbit ileum in the Ussing chamber studied under

increased Isc, PD, Gt, and net Na absorption which accounts for 70% of the increased in Isc. Removal of HCO_3^- from Ringer's solution did not alter any parameters but increased net Cl secretion due to a decrease in mucosal-to-serosal Cl flux. Reduction of 50% of the [Cl] in HCO_3^- -free solution decreased the net Cl secretion to the level in Ringer's solution and increased net Na absorption. Removal of Cl decreased Isc to the value of net Na absorption and decreased the Na influx across the mucosal membrane by 39%. Isc and PD were near zero and net Cl absorption was observed in a Na-free solution. These results are consistent with the transport mechanism that consists of 1) an electrogenic Na absorptive process that accounts for the Isc, 2) a neutral NaCl coupled secretory process, and 3) a system by which HCO_3^- secretion exchanges for Cl^- absorption.(21)

The mechanisms whereby methylprednisolone exerts its effect on electrolyte transport in rat ileum were investigated in the following studies. Administration of the glucocorticoid methylprednisolone, 3 mg/100 g of body weight for three days, in intact rats increased intestinal mucosal guanylate cyclase and Na-K-ATPase activities, short-circuit current (Isc), electrical potential difference (PD), net Na absorption and net Cl secretion and reversed HCO_3^- transport from secretion to absorption in isolated ileum. At 6 hr after a single dose of methylprednisolone, stimulation of guanylate cyclase activity was already maximal whereas Na-K-ATPase activity was not altered. The associated changes in intestinal transport properties with the increased guanylate cyclase activity were an increase in Isc and PD and a reversal of net Cl absorption to net Cl secretion. These results suggest that an initial response to methylprednisolone administration is a persistent increase in intestinal guanylate cyclase activity that mediates an increase in Isc and Cl secretion, then followed by a superimposed effect of increased Na-K-ATPase activity that mediates an increase in net Na absorption. A third effect of methylprednisolone may be the reversal of net HCO_3^- transport and its mechanism is not known.(4)

A single intramuscular (IM) dose of the glucocorticoid methylprednisolone (3mg/100g) significantly increased rat jejunal and ileal mucosal guanylate cyclase (GTP pyrophosphatylase (cyclizing); EC 4.6.1.2) activity and cyclic GMP (cGMP) concentration 6 hours after injection, was not associated with changes in mucosal cyclic AMP concentration, and occurred before any increase in Na-K-activated adenosine triphosphatase activity could be detected. In contrast to the findings in the small intestine, no alterations in guanylate cyclase activity

short-circuited conditions. 5HT exposure to rabbit ileum caused two effects: 1) a transient increase in short-circuit current (Isc) and 2) neutral NaCl secretion. The 5HT-induced increase in Isc began within 30 seconds of 5HT addition; peaked and returned to baseline within 2 minutes; occurred following serosal but not mucosal addition of 5HT; was dose dependent, with the threshold effect at 2.6×10^{-6} M, maximal effect at 2.6×10^{-6} M and half maximal effect at approximately 10^{-6} M; did not affect the glucose-dependent or theophylline-dependent Isc response. The 5HT-induced change in Na and Cl transport consisted of decreased mucosal to serosal and increased serosal to mucosal fluxes with production of 0 net Na movement and net Cl secretion; the effect on net Na and Cl transport was equal with no change in residual ion flux, tissue conductance or Isc during the period of flux determinations. Serosal 5HT altered net Na and Cl fluxes but mucosal 5HT was without effect. The 5HT-induced effect on Na and Cl fluxes was dose dependent with the same threshold, maximal and half maximal concentrations as the Isc. The 5HT effects could be completely and immediately reversed by removing the serosal 5HT. To determine whether these effects of 5HT were calcium (Ca) dependent, Ca was removed from the serosal surface. Removal of serosal Ca totally prevented the effect of 5HT on both Isc and on net Na and Cl fluxes and could be reversed by readdition of Ca. These findings suggest that 5HT causes active ileal secretion of Na and Cl which is a) neutral; b) reversible; c) dose dependent; and d) dependent on serosal Ca. The fact that such a low concentration of 5HT alters electrolyte transport suggests that 5HT might be a physiologic regulator of ileal electrolyte transport.(24)

Serotonin was shown to inhibit active neutral NaCl absorption by studying active electrolyte transport in epithelial sheets of rabbit ileum, gallbladder and colon under short-circuited conditions. Serotonin added to the serosal surface of rabbit ileum caused a dose-dependent equal effect on net Na and Cl fluxes which consisted primarily of inhibition of mucosal-to-serosal fluxes. A threshold effect occurred at a serotonin concentration of 2.6×10^{-8} M; maximal effect at 2.6×10^{-8} M; and the concentration for the half-maximal effect on net Na flux was 2.4×10^{-7} M and for net Cl flux was 4.0×10^{-7} M. The serotonin effect was not associated with alterations of the short-circuit current during the period of electrolyte flux determinations and was not associated with any changes in residual ion flux. Consistent with this inhibition of neutral NaCl absorption demonstrated in rabbit ileum, serotonin induced equal inhibition of net Na and Cl absorption in rabbit gallbladder but had no effect on rabbit colon. Serotonin did

not alter all aspects of ileal absorptive function since serotonin did not alter glucose-dependent Na absorption. The serotonin effect was dependent on an intact Na-K-ATPase since pretreatment with ouabain (10^{-4} M) prevented any subsequent effect of serotonin on electrolyte transport. In addition, cAMP and serotonin both altered the neutral NaCl absorptive process since following stimulation of ileal secretion with a maximum concentration of theophylline, addition of serotonin did not cause any further effect, but when rabbit ileum was exposed first to a maximum concentration of serotonin and then exposed to theophylline, theophylline caused additional electrogenic Cl secretion.(5)

The mechanism of action for Shigella Toxin (ST) mediated intestinal secretion had not been clearly defined. Rabbit ileal loops were incubated 4 hrs with either a control solution or a dose of ST standardized by HeLa cell assay. The loop was excised, the muscularis mucosae removed and the tissue mounted in Ussing chambers. The short-circuit current (I_{sc}), potential difference (P_d) and conductance (G) were monitored. The mucosal (m)-to-serosal (s) and s-to-m fluxes for Na and Cl were determined using isotopes and the net and residual ion (J_r) fluxes calculated. One set of experiments used a bathing solution of Ringers- HCO_3 and another used a Cl-free solution. The effect of ST in Ringers was to produce a net secretion of Na and Cl. This was solely the result of an increase in their s-to-m fluxes. The magnitudes of the changes in the Na and Cl net fluxes were equal, as were the increases in their s-to-m fluxes. There was no change in m-to-s flux, I_{sc} , P_d , G or J_r . The secretory effect of ST was abolished in the Cl-free solution, there being no changes in any of the parameters as compared with the Cl-free controls. These results are most consistent with the stimulation of a neutral, coupled Na-Cl secretory system. Therefore it appears that ST has a distinct mechanism of action as compared with theophylline, cyclic-AMP, Cholera toxin, etc.(9)

Patients with intestinal obstruction can have intraluminal accumulation of fluid and increased intraluminal hydrostatic pressure (IHP), however available information on the direct effect of IHP on intestinal water and electrolyte transport in vivo remains incomplete. In this study jejunal, ileal, or colonic IHP was acutely increased in vivo in male New Zealand white rabbits, and the effect was determined on water and electrolyte transport in the test segment and adjacent control segment using an in vivo perfusion system with ^{14}C -polyethylene glycol as a nonabsorbable water marker. Test segment IHP was increased by raising the efflux catheter 10 to 70 cm above the

abdominal surface, while control segment IHP was held constant at 0 cm water. Acutely increased IHP in the jejunum and ileum caused a quantitatively similar pressure dependent decrease in net water and electrolyte absorption in test segments, but no significant change in control segment transport. Increased IHP did not significantly alter colon transport. Since basal absorption in the jejunum exceeded that in the ileum, the IHP-induced loss of absorption produced net secretion only in the ileum. This secretory process was linearly dependent on lumen pressure, isotonic, and at low IHP, reversible. High IHP was associated with tissue hemorrhage, epithelial sloughing and poor reversibility of transport changes. IHP-induced secretion was not dependent on significant systemic effects of luminal distention, or at low IHP, loss of epithelial integrity. Intraluminal accumulation of fluid seen in intestinal obstruction may be related to the secretion induced by increased IHP.(6,26)

Acutely increased intraluminal hydrostatic pressure (IHP) up to 70 cm water in rabbits is associated with locally produced secretion of water and electrolytes in the ileum, decreased absorption in the jejunum, and no transport changes in the colon. The mechanism of these phenomena was investigated by studying active and passive transport processes.

The effect of acutely increased IHP on variables associated with active intestinal transport was studied in the ileum. It was found that acutely increased IHP did not significantly alter specific mucosal adenylate cyclase or Na-K-ATPase activities, did not alter mucosal active ion transport (as evidenced by in vitro short circuit, current), and did not prevent the in vivo stimulation of water and electrolyte absorption by luminal glucose. Secretion of water and electrolytes is not due to alterations in these processes occurring at the lateral, basal, and luminal brush border cell membranes. Luminal glucose may have a role as adjunctive therapy for IHP-induced intestinal secretion.

The effects of acutely increased IHP on the following variables associated with passive intestinal transport was studied: unidirectional water transport, erythritol and mannitol clearance from plasma to lumen, tissue architecture, and in the ileum, tissue blood flow rate. Acute moderate increases in IHP caused: 1) increased effective mucosal surface area in the small intestine, as evidenced by increased unidirectional water transport, and gross intestinal distention with marked separation of mucosal villi, 2) increased tissue permeability, as evidenced by a proportionately greater increase in mannitol

(Stokes radius 0.4 nm) than in erythritol (Stokes radius 0.32 nm) clearance. In the ileum higher IHP was associated with decreased unidirectional water transport and mannitol loss of anatomical mucosal surface area. In the ileum increased IHP did not significantly change mucosa-submucosa and muscularis-serosa blood flow, although mucosal hemorrhage was evident at the highest IHP. Increased IHP caused less intestinal distention in the colon, compared to the small intestine, and no significant change in effective surface area or tissue permeability, as indicated by unchanged unidirectional water transport and mannitol and erythritol clearances. Altered intestinal transport due to acutely increased IHP is apparently mediated by increased effective mucosal surface area and/or increased tissue permeability. Integration of these results with published literature suggests that increased IHP can alter the hydrodynamics of the mucosal microcirculation to produce a significant driving force for passive filtration-secretion through mucosal tissue having augmented effective surface area and permeability.(7,26)

A method was described for measuring ileal blood flow in the anesthetized (pentobarbital sodium) rabbit by the intraventricular injection of microspheres (15 μ m) labeled with cerium-141 or chromium-51; with this method the amount of labeled microspheres lodging in the tissue is proportional to the blood flow. Blood flow to the ileal mucosa plus submucosa could be separated from flow to the ileal muscularis propria plus serosa by this technique. Simultaneous and sequential injections of radiolabeled microspheres gave similar measurements of ileal blood flow and did not affect ileal water absorption. Increasing ileal water absorption by treatment with the glucocorticoid methylprednisolone (3 mg/100 g per day for 3 days) increased blood flow to both compartments of the ileum and also to the colon, liver, and kidneys; methylprednisolone treatment did not alter blood flow when studies were performed before the methylprednisolone-induced increase in ileal water absorption had occurred. In contrast, intestinal secretagogues that induced both active ileal secretion (purified cholera toxin and serotonin) and passive ileal secretion (hypertonic mannitol) did not affect ileal blood flow. These studies indicate that increased ileal water absorption is associated with increased ileal blood flow, whereas intestinal secretion is not necessarily associated with an alteration in ileal blood flow.(27)

The ability of the beta-adrenergic receptor antagonist propranolol to effect intestinal secretion induced by active and passive mechanisms was evaluated in the rat ileum and colon.

Ileal and colonic loops were exposed to cholera toxin, dibutyryl cAMP, and hypertonic mannitol in vivo to induce water and electrolyte secretion. When studied 3.5-4.5 hr after cholera toxin inoculation, water and electrolyte secretion and increased adenylate cyclase activity and cyclic AMP content were observed in intestine from untreated animals, but Na-K-ATPase, Mg-ATPase, and cyclic nucleotide phosphodiesterase were unaffected. Treatment with D,L-propranolol, SC 4 mg/kg/day for 3 days, did not affect water or electrolyte movement or intestinal enzyme activities or cyclic AMP content in saline-inoculated loops, but significantly reduced the cholera toxin-induced ileal water and electrolyte secretion and completely prevented the cholera toxin-induced colonic secretion. Propranolol treatment did not alter the cholera toxin-induced increase in adenylate cyclase activity or cyclic AMP content. Similar treatment with D,L-propranolol failed to affect ileal water secretion induced by hypertonic mannitol. Treatment with D-propranolol, SC 4 mg/kg/day for 3 days, did not affect the ileal secretion induced by cholera toxin inoculation. To document the site of propranolol inhibition of intestinal secretion, secretion was induced by ileal perfusion with dibutyryl cAMP. Propranolol treatment completely prevented this dibutyryl cAMP-induced ileal secretion. These results indicate that D,L-propranolol is able to prevent cholera toxin-induced ileal and colonic secretion in the rat but does not affect passive secretion caused by hypertonic mannitol. This action of propranolol is stereo-specific and is not associated with inhibition of cholera toxin-induced activation of adenylate cyclase or increase in cyclic AMP content and apparently involves a step in the intestinal secretory process after activation of adenylate cyclase.(28)

A paper on the mechanism of Fluid and electrolyte secretion in the germ-free rat cecum was published. The cecum of the germ-free rat is filled with a large volume of liquid: cecum plus cecal contents comprise up to 25% of the rodent's body weight. To explain the presence of the liquid cecal contents in the germ-free rat, cecal transport of water and electrolytes was studied using a closed-loop technique with [14 C]polyethylene glycol as a nonabsorbable marker. When NaCl (154 mEq/liter) was instilled into germ-free rat ceca, absorption of water, Na, and Cl occurred and was similar to that in conventional animals. In contrast, when an equal volume of supernatant from germ-free cecal contents was instilled into germ-free rat ceca, secretion of water, Na, and Cl occurred. Similarly, when the germ-free supernatant was instilled into ceca of conventional rats, secretion of water, Na, and Cl occurred at rates equal to that seen in the germ-free rat.

Therefore, it appeared that the composition of the germ-free cecal contents was primarily responsible for the cecal secretion. Analysis of the germ-free cecal contents to determine what caused the cecal secretion revealed very low measurable anions (Cl^- 2, HCO_3^- 2 mEq/liter), a low Na concentration, slight hyperosmolality as determined by freezing-point depression, and elevated colloid osmotic pressure. Fluid made up with Na_2SO_4 to resemble the germ-free cecal supernatant in ionic composition and absence of exchangeable anions produced cecal secretion equal to that of the germ-free rat cecal contents. Fluid simulated to reproduce the effects of the low Na concentration and also the colligative properties and colloid osmotic pressure had minimal effects on cecal transport. These studies demonstrate that cecal enlargement in the germ-free rat is associated with cecal secretion of water and electrolytes, that germ-free ceca can transport water and electrolytes in a normal fashion, and that cecal secretion in the germ-free rat is due primarily to the absence of permeable anions in the germ-free cecal contents.(40)

D. Alterations in Intestinal Motility.

Migrating action potential complexes (MAPC) have been described in rabbit ileal loops injected with cholera toxin (CT), ricinoleic acid, and E. coli toxins. The MAPC is a reproducible, propagated burst of myoelectric spike activity that occurs several hours after the secretory activity of CT. To examine whether MAPC activity is independent of the fluid secretory activity of CT, we injected ligated rabbit ileal loops with 1ml of 1 of 5 solutions: purified CT (100ug), cholera toxinoid (CD) (100ug), cholera antiserum (AS), purified CT+AS, or EDTA control. As in the previous studies with this model, electrodes were sewn to the distal ileal serosa at 2.5 cm intervals and the animal recorded for 6 hr after injection. Fluid secretion was used as a measure of CT activity. Rabbits remained at 39°C during the study. Slow wave activity was essentially the same in all groups throughout the study. CD, a nonpathogenic aggregate of B subunits of CT produced a similar number of MAPC (13 ± 2.2) during the 6 hr observation period as CT (13.7 ± 2.7). These values were significantly different ($p < 0.01$) from controls. MAPC onset time with CD (181 min after injection), was slightly later than that seen with CT (127 min after injection), but the propagation velocity of the MAPC was essentially the same in both groups (1.3 cm/sec vs 1.5 cm/sec) CT injected animals secreted 4.6 ± 0.5 ml of fluid during the observation period. None of the other solutions produced measurable fluid secretion. To neutralize Cl^- , AS was incubated with CT to provide slight antibody excess. Incubated AS+CT

produced fewer MAPC than CT, and the mixture had no CT activity in the adrenal cell assay. We separated AS+CT into a precipitate and a supernatant; neither produced CT activity in the adrenal cell assay. The supernatant gave 17 ± 3 MAPC during the 6 hr period, slightly more than CT. The precipitate produced fewer events (3 ± 1) than CT or the supernatant ($p < 0.02$). We concluded: 1) MAPC activity of CT is independent of fluid secretion, 2) MAPC activity is not blocked by neutralization of fluid secretion or adrenal cell assay activity of CT with AS, 3) MAPC activity is most likely produced by the B subunit of CT.(29)

A previous study from this laboratory showed that the cholera-induced migrating action potential complex (MAPC) was caused by the B subunit of cholera toxin (CT). We examined acute rabbit ileal loops exposed to CT subunits, pure CT B subunits, and two modified CT derivatives (modified CT and modified B.) The subunit was modified by reaction of its single tryptophan residue with nitrophenylsulfonyl chloride. These modified compounds have no binding activity to membrane sites. A 20 cm loop of distal ileum was isolated in 2-3 kg rabbits. Proximal and distal catheters were placed in the loop for injection and drainage. 3 monopolar Ag-Ag(Cl)₂ electrodes were sewn to the serosa of the loop at 3-4 cm intervals. After baseline recording, 1 solution (100 ug/ml) was injected into the proximal catheter: CT, modified CT, B subunits, modified B, or A subunits. Records were read for MAPC during the 6-hr study. Fluid output confirmed toxin activity in the loop; Y_1 adrenal cell assay was done on paired coded samples. CT produced 13.2 MAPC during the period with 22 ml of fluid. B subunits produced 13.7 MAPC and no fluid. Modified CT produced 1.3 MAPC, while modified B and A subunits produced no MAPC and no fluid output. All modified solutions and A subunits produced significantly fewer MAPC than CT or B ($p < 0.05$). Modified CT produced significantly less fluid than CT ($p < 0.02$). Y_1 assay was positive with rounding of all cells at 3 pg CT, while all other solutions were negative at 100-fold greater concentrations. We conclude: 1) CT B and not A subunits produce MAPC-activity, 2) modification of B subunits to eliminate membrane binding, eliminates MAPC-activity, 3) modification of the B subunit decreases Y_1 activity by 100-fold.(30)

Other studies on the effects of prostaglandins on esophageal smooth muscle were reported. Previous studies from this laboratory suggested that prostaglandins (PG) mediate LES hypotension associated with inflammation. To examine this relationship, PGE_1 was infused arterially above the celiac axis in cats to produce LES hypotension similar to experimental

esophagitis. During PG infusion, the response to an IV bolus of edrophonium or urecholine was measured to determine whether cholinergic or smooth muscle function was inhibited by the PG. LES pressure was recorded continuously using a perfused sleeve catheter. After baseline pressure, PGE₁ (0.15 ug/kg/min) was infused for 30 min. At the nadir of LES hypotension (15 min), an IV bolus of either urecholine (25 ug/kg) or edrophonium (100 ug/kg) was given. During PGE₁ infusion, LES pressure decreased by 33 mmHg. In contrast, LES pressure rose 29 mmHg above baseline after urecholine injection. Although PGE₁ significantly decreased LES pressure (p 0.05), the pressure rise after urecholine was not blocked by the prostaglandin. Urecholine increased LES pressure by 62 mmHg compared to the nadir of PGE₁-induced hypotension (p 0.001). In contrast, the LES pressure rise with edrophonium in control animals was blocked by PGE₁ infusion in tested animals. We conclude: 1) PGE₁ infusion does not affect the LES pressure rise after urecholine, but blocks the LES response to edrophonium, 2) LES response to these medications during PGE₁ infusion is similar to results seen in cats with experimental esophagitis. This study suggests that prostaglandins and inflammation act on the LES through similar mechanisms, that is, by inhibiting cholinergic regulation of LES pressure while leaving smooth muscle function unaffected.(31)

Previous studies demonstrated the hypotensive effect of IV bolus doses of PGE₁ and PGE₂ on the lower esophageal sphincter (LES). We examined the effect of low dose continuous IV infusion of PGE₁, PGE₂, PI, or 6-keto-PGF_{1α} (all at 0.15 ug/kg/min) on LES pressure in sedated cats. Esophageal sphincter pressure was recorded continuously during the 60 min study using a perfused sleeve catheter. After a baseline pressure recording, 1 of 6 solutions (PGE₁, PGE₂, PI, 6-keto-PGF_{1α}, 0.9% saline, or Tris buffer) was infused in a hindquarter vein at 1.1 ml/min for 30 min. Tracings were read blindly for LES pressure values. PGE₁ progressively decreased LES pressure during the infusion to a nadir of 17±10 mmHg below baseline at 15 min, significantly different from control (p 0.05). PGE₂ increased LES pressure throughout the study, but there were no significant differences from saline control. Tris (pH 10.5), [the solvent for PI and 6-keto-PGF_{1α}], increased sphincter pressure during the infusion to a peak of 18.4±8.8 mmHg above baseline at 25 min. 6-keto-PGF_{1α} produced a similar progressive rise in LES pressure. PI however, decreased LES pressure during the infusion with significant differences from Tris control at 15 min (p 0.05), and 30 min (p 0.05), the end of the infusion. After PI infusion was stopped, LES pressure promptly rose and paralleled the curve seen with

Tris control. We concluded: 1) Continuous IV PGE_1 produces significant LES hypotension, 2) Continuous IV PI, but not its major metabolic product, 6-keto- $\text{PGF}_{1\alpha}$, also produces LES hypotension.(32)

Intravenous prostaglandins (PGE_1 , PGE_2) decrease lower esophageal sphincter pressure (LESP), yet these compounds are nearly completely degraded in the cat lung in a single passage. We compared continuous arterial and venous infusions of three prostaglandins for possible differences in their effect on LESP in cats. Arterial infusions were given 1 cm above the diaphragm and LESP was determined by a perfused sleeve catheter positioned to allow continuous pressure recording during the 30 min infusion and for an additional 30 min. After a baseline period, 1 of 5 solutions was infused: PGE_1 , PGE_2 (all at 0.15 ug/kg/min), 0.9% saline, or Tris buffer. Records were read blindly and LESP determined for each minute. During arterial PGE_1 , LESP fell in the first 5 min, reaching a nadir of 34 ± 1.3 mmHg below baseline ($p < 0.001$) at 30 min, the end of the infusion. Arterial PGE_1 caused a greater fall in LESP than venous PGE_1 , both at the end of the 30 min infusion ($p < 0.05$) and at the end of the 30 min post-infusion period ($p < 0.01$). Control infusions were not associated with significant changes in LESP from baseline at any time period. LESP reached a plateau after 30 min of arterial PGI_2 of 10 ± 3 mmHg below baseline, but rose within 1 min post-infusion to a significant peak 16 ± 5 mmHg above baseline at 30 min post-infusion ($p < 0.01$). We conclude: 1) Arterial PGE_1 causes a significantly more pronounced and more prolonged fall in LESP than venous PGE_1 , 2) Arterial PGI_2 lowers LESP slightly during infusion, but significantly raises LESP above baseline post-infusion, 3) Arterial or venous PGE_2 causes no significant change in LESP during continuous infusion. 4) Arterial and venous PGE_1 , and not PGE_2 or PGI_2 change LESP to a different magnitude in the cat.(33)

II Liver Fibrosis (34), Using Schistosomiasis as a Model System.

Work in our section has focused on two areas. The first is detailed examination of granulomas, the site of liver collagen synthesis in mice with Schistosoma mansoni infection. The second is progression, reversibility and pharmacologic therapy of liver fibrosis in the rabbit with Schistosoma japonicum infection, an animal model that more closely resembles chronic hepatosplenic schistosomiasis in humans.(35)

A. Granulomas.

The major relevance of the mouse infected with Schistosoma mansoni to human schistosomiasis is in its reproduction of granulomas around schistosome eggs, thought to be the initial step leading to later irreversible changes. Earlier, we found that these granulomas, isolated from the livers of mice 8 to 10 weeks after a 50-cercaria infection, contained all of the newly-deposited liver collagen and retained the bulk of the in vitro collagen-synthesizing activity present in intact liver slices. Others showed that these granulomas elaborated material that stimulated division of fibroblasts, and we showed that production of collagenase by these granulomas is considerable. During the past year we evaluated the organ-specificity of fibrosis in these granulomas, and examined the metabolism of proline in the granulomas.

1. Organ Specificity of Fibrosis.

After subcutaneous infection with S. mansoni cercariae, there is heavy deposition of eggs in the liver and intestine by 9 weeks. The size and cellularity of granulomas around eggs in the small intestine and liver appear similar. After washing the lumen and removing villus tips by scraping, the remaining eggs in the small intestine are in granulomas in the muscle and submucosal layers. Tissue digests showed similar egg counts in small intestine and liver. Collagen content of infected liver, measured as hydroxyproline content, was markedly increased compared with that of normal liver, as expected from earlier work. In contrast, however, the collagen content of infected intestine was no greater than that of normal intestine. We then measured collagen biosynthesis in vitro by incubating tissue slices with ^{14}C proline and following formation of nondialyzable hydroxyproline. As expected, there was a striking increase in collagen biosynthesis in the infected liver slices, compared with normal. However, there was only a minimal increase in collagen biosynthesis in infected intestine slices compared with normal. We concluded that in response to the same degree of granulomatous inflammation, fibrosis in the liver was far greater than that in the small intestine.

To start to examine possible reasons for this organ-specificity of the fibrotic response, we measured free proline levels in both the liver and intestine. As we found earlier, there was an approximately 2-fold increase in liver free proline in the infected animals, consistent with the idea that an increased

supply of this substrate is permissive for maximal collagen peptide synthesis in a tissue that is "turned on" to this process. Intestinal free proline in the infected animals was no different from normal. When infected liver slices are incubated with increasing concentrations of free proline, there is a sharp increase in collagen peptide synthesis up to a saturating maximum level. Increasing the free proline in intestinal slice experiments even to very high levels led to only a modest increase in collagen synthesis in vitro. In infected intestine, both the lack of free proline accumulation and the relative inability to use added proline for collagen formation suggest that the granulomas in this tissue do not contain the machinery to make an intense fibrotic response to the degree seen in infected liver.

We next produced granulomas in the lungs of mice, using the method of partial ligation of the portal vein, leading to porta-systemic collateral formation. As in the intestine, we noted similar formation of granulomas around eggs in the lungs, and heavy egg deposition. Also similar to the intestine, there was only a minimal increase in infected lung collagen content and no difference in collagen synthesis in vitro with infected lung slices, as compared with normal.

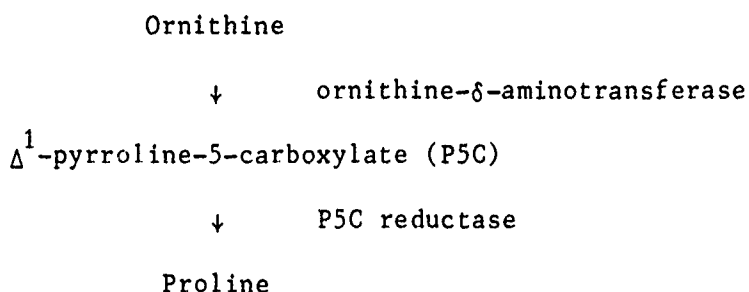
These findings all suggest that there may be unique characteristics of liver granulomas in this model that lead to much more fibrosis than in the granulomas of other organs in response to an identical antigenic stimulus. The sorting out of such differences is a formidable task that we are starting to approach with further metabolic studies, ultrastructural examination and cell isolations. We hope that the organ specificity of the effector response, fibrosis, will be a useful tool in evaluating the importance of the many humoral and cellular components of the inflammatory process that might have a specific relationship to fibrosis.(8,36)

2. Proline Trapping in Granulomas.

As mentioned above, free proline supply is an important permissive factor for collagen peptide synthesis in a tissue "turned on" to this process. We found earlier that the likely source of free proline in normal and infected mouse and human liver was metabolism of arginine to proline. Hepatocytes are known to actively form and degrade proline. Proline is known to be a major nitrogen excretion product of adult trematodes, and the possibility that schistosome eggs within granulomas make proline is of interest. There have been no studies of proline metabolism in liver granulomas. In earlier

studies, our inability to localize free proline pools to compartments where collagen synthesis is occurring has made it difficult to go beyond general statements about proline supply in the whole organ. The following data suggest that there may be an important proline-supply relationship in fibrosis between the liver cells and the granulomas.

Arginine is rapidly cleaved to ornithine in liver, and two sequential enzymes convert ornithine to proline:(37)



The interconversion between proline and P5C, a cyclic ring compound, is a reversible oxidation/reduction. A separate enzyme, proline oxidase, catalyzes the conversion of proline to P5C. P5C can be further metabolized by another enzyme, P5C dehydrogenase.

We assayed these enzymes in infected mouse liver, measuring their activities in isolated granulomas and in liver supernate after removal of the granulomas by sedimentation. Activities of all 4 enzymes were far greater in the liver than those in the isolated granulomas. Enzymes from the liver and the granulomas had the same optimum activity characteristics and the same subcellular localizations on sucrose centrifugation as the enzymes prepared from normal mouse liver. While there was some activity of the proline-synthesizing enzymes in isolated granulomas, proline-degrading activity was only present as small levels of proline oxidase and no detectible P5C dehydrogenase.

We previously showed that collagen peptide synthesis in vitro was maximal in isolated granulomas, and that added ^{14}C proline in vitro readily enters the granulomas and equilibrates with the intracellular proline. Thus it seems likely that much of the proline used for collagen peptide synthesis originates from the liver cells and enters a new metabolic environment in the granulomas, where it is avidly used for collagen peptide synthesis and where the potential for proline degradation is minimal.(9,38)

We still cannot measure proline pools and fluxes within the granulomas, and the possibility that there is metabolic proline channeling in different compartments within the granulomas has to be addressed. Further understanding of the regulation of liver collagen synthesis at the substrate level, and the design of effective countermeasures (e.g. proline analogues) depend on extending our studies of proline handling within the granulomas and on matching up proline-metabolizing and collagen-synthesizing compartments.

B. Chronic Hepatosplenic Schistosomiasis In Rabbits.

Rabbits infected with up to 500 Schistosoma japonicum cercariae provide an experimental model of chronic pipestem fibrosis that resembles the fibrosis of chronic human schistosomiasis more closely than does the S. mansoni-infected mouse. In collaboration with Dr. Allen Cheever at NIH, we are conducting a longterm study aimed at defining the potential for reversibility of such chronic disease. Investigators at NIH, confirming original findings of Japanese workers, have shown that liver collagen content increases markedly in S. japonicum-infected rabbits and that a histologic picture of Symmers' fibrosis is present in most animals 20-30 weeks after infection. Later, the collagen content and histologic appearance of fibrosis regress somewhat, so that by 50 weeks after infection there is evidence of advanced, quiescent, apparently stable disease.

We have found marked increases in liver free proline and collagen biosynthesis with infected liver slices in this model, coinciding with maximal appearance of disease activity at 20-30 weeks. In agreement with other evidence of progression of the disease to a chronic phase, there is a decrease of collagen-synthesizing activity of liver slices to only twice normal by 50 weeks.

We are now examining the potential for reversibility of this lesion after parasitologic cure. Twenty weeks after infection, rabbits undergo laparotomy and wedge liver biopsy. The collagen content, egg count, free proline content, and in vitro collagen-synthesizing capacity of liver slices from the biopsy specimens are measured by the same methods we used in our earlier studies of mouse and human liver. Rabbits are then assigned to three groups matched for the extent of Symmers' fibrosis by histologic examination, and for degree of fibrosis as determined chemically. Two of the groups are cured of their infection and the third group serves as a control. After parasitologic cure, one of the groups is begun on daily

intraperitoneal injections of colchicine in a maximum-tolerated dose (0.5 mg daily) and the other groups are begun on intraperitoneal saline injections. Extent of disease will be reassessed by the above methods at a second biopsy 40 weeks after infection, and again at 60 weeks.(10)

From this study, we hope to be able to assess the potential for reversal of early pipestem fibrosis after parasitologic cure of infection, and to plan studies of tissue collagenases in this model.(39) In addition, we hope to learn whether colchicine, a compound that may favorably affect the course of alcoholic cirrhosis, has any therapeutic potential in promoting resolution of Symmers' fibrosis. We also hope to obtain additional information which will guide our design of studies of reversibility of this process in humans.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ⁶	2 DATE OF SUMMARY ⁶	REPORT CONTROL SYMBOL	
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a. PRIMARY	62770A	3M162770A802		00		006	
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23 TECHNICAL OBJECTIVE ⁶ 24 APPROACH, 25 PROBLEM (Provide individual paragraphs identified by number. Provide text of each with Security Classification Code)							
23. (U) Develop experimental rickettsial immunogens; define the pathology of rickettsial infections in laboratory animals; determine the sequence of events leading to immunity following vaccination or infection. These studies are aimed directly at development of safe, efficacious rickettsial vaccines that will protect deployed military troops, and development of accurate, sensitive immunoassays to evaluate the extent of immunity induced by vaccination.							
24. (U) Gamma irradiation of rickettsiae to produce attenuated immunogens. Evaluate different strains and combinations of strains for use as immunogens to provide long-lasting, broad-spectrum protection against Rickettsia tsutsugamushi infection. Analyze in vitro the effects of lymphocytes and lymphocyte products on macrophages. Determine the genetic basis of resistance and sensitivity of the mouse model to scrub typhus infection							
25. (U) 78 10 - 79 09 Broad spectrum protection against scrub typhus infection in mice was achieved using combinations of 3 strains of rickettsiae. Trivalent mixtures of Karp Gilliam, and Kato rickettsiae exhibited a striking synergistic effect on the immune response of mice. Detection of residual, replicating rickettsiae in inadequately irradiated immunogen suspensions was most effectively accomplished by transfer of spleen cell homogenates from vaccinated to unvaccinated mice. Cultures of mouse macrophages activated with lymphokines showed a significant increase in rickettsia-free cells after exposure to Rickettsia tsutsugamushi. Activated macrophages also yielded 60 percent fewer viable, intracellular rickettsiae. Rickettsiacidal activity was mediated by decreased infectivity of rickettsiae and/or increased intracellular killing of rickettsiae. Natural resistance of mice to lethal scrub typhus infections was shown to be controlled by a single, autosomal, dominant gene (Ric) on chromosome 5. The Ric locus was closely linked to the retinal degeneration locus. For technical report see Walter Reed Army Institute of Research Annual Progress Report. 1 Oct 78 - 30 Sep 79.							

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 006 Rickettsial Diseases of Military Personnel

Investigators:

Principals: Joseph V. Osterman, PhD; LTC Michael G. Groves, VC; LTC Edward H. Stephenson, VC; MAJ George H. G. Eisenberg, Jr., MSC; Carol Nacy, PhD

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Description:

Investigations to develop experimental rickettsial immunogens, define the pathogenesis of rickettsial infections in laboratory animals, and determine the sequence of events leading to immunity following vaccination or infection. These studies are directed toward development of safe, efficacious rickettsial vaccines which will protect deployed troops, and development of accurate, sensitive immunoassays to evaluate the extent of immunity induced by vaccination.

During the reporting period, research activities have included: (1) scrub typhus vaccine development, (2) host immune response to Rickettsia tsutsugamushi infection and vaccination, and (3) natural resistance to scrub typhus infection.

Progress:

1. Gamma-irradiated scrub typhus immunogens: broad spectrum immunity with combinations of rickettsial strains.

Epidemiological investigations have revealed the existence of numerous strains of Rickettsia tsutsugamushi that can be differentiated on the basis of serological tests and virulence characteristics in laboratory animals. In some regions endemic for scrub typhus, a single strain appears to be dominant (1), but other reports indicate that several different strains may coexist in rather small geographical areas (2,3,4). This antigenic diversity has been a major consideration in the development of a scrub typhus vaccine. Although single-strain formalinized rickettsiae have been shown to elicit significant levels of protection against homologous strain challenge in laboratory animals (5), gamma-radiation inactivated scrub typhus immunogens protected mice against challenge with both homologous and heterologous strains of R tsutsugamushi (6). Subsequent studies from this laboratory (7) defined the optimum temporal regimen for

vaccination, and examined the development and duration of the protective immune response in BALB/cDub (BALB) mice. Animals vaccinated with gamma-irradiated Karp immunogen were resistant to a 10^4 MLD₅₀ homologous challenge for 9 months. However, resistance to the heterologous Kato strain waned rapidly, indicating the necessity to prolong effective heterologous protection and to assess resistance against other strains of scrub typhus rickettsiae. Such a study was facilitated by the recent discovery that C3H/HeDub (C3H) mice are susceptible to lethal infection with strains of R tsutsugamushi to which BALB mice are naturally resistant (8).

In this study, we determined the comparability of data obtained with BALB and C3H mice, then used the latter animal model to allow expansion of the number of rickettsial strains employed for challenge and preparation of immunogens. In addition to Karp and Kato, we have used Gilliam, the third of the classical prototype strains of R tsutsugamushi (9); Buie, a strain shown to produce broadly cross-reactive antisera in rabbits (10); and Kostival, one of the more effective formalinized immunogens in cross-vaccination studies (5). These immunogens were used as single strains and 3-strain combinations, concluding with a one year study that assessed the durability and scope of scrub typhus immunity in vaccinated mice and contrasted this protection with the durability of heterologous strain immunity occurring after sublethal infection.

Mice. Female BALB and C3H mice (Flow Laboratories, Dublin, VA), 18 to 22 gms, were used throughout this study.

Rickettsiae. The Karp, Gilliam, Kato, Kostival, and Buie strains of R tsutsugamushi were propagated, stored, and quantified using methods previously reported (11). Only those suspensions having titers $\geq 10^8$ 50% mouse lethal doses (MLD₅₀)/gm of yolk sac, as determined in susceptible mice, were used. The 50% end points were calculated from mortality data by the Spearman-Kärber method (12).

Immunogens. Radiation-inactivated immunogens were prepared by exposing frozen 20% yolk sac suspensions to 300 Krad of gamma radiation in a ⁶⁰Co gamma irradiator (Gamma Cell 220, Atomic Energy of Canada Limited, Ottawa, Canada) as previously described (6). Immunogen mass was quantified in MLD₅₀ units based on mouse titration of unirradiated organisms.

Vaccination and challenge. All injections were administered intraperitoneally (i.p.) in a standard volume of 0.2 ml. Immunogenicity of irradiated suspensions was determined by administering a 1,000 MLD₅₀ homologous strain challenge dose to groups of mice vaccinated 24 days previously with a single dose of immunogen

diluted in 10-fold increments over an appropriate dose range. The 50% protective dose (PD₅₀) was expressed in MLD₅₀ units by calculating the difference between the log₁₀ of the 50% protective dilution and the log₁₀ of the MLD₅₀ of the unirradiated suspension (6). The level of immunity, or immunity index, induced by vaccination according to the standard regimen of 3 injections at 5-day intervals was determined by challenge titration in vaccinated and control mice on day 31. The immunity index was calculated by subtracting the log₁₀ of the MLD₅₀ in vaccinates from that in controls (13). For comparison of vaccination methods or immunogen combinations when multiple-strain challenge was employed, composite immunity indices were obtained from mean MLD₅₀ values calculated after summing mortality data for all challenge strains at each challenge dose.

Effect of gamma radiation on lethality and immunogenicity of rickettsiae. Since we used the Karp strain of R tsutsugamushi and BALB mice for most of our previous scrub typhus immunogen studies (6,7), it was necessary to determine if other rickettsial strains were inactivated by gamma radiation in a manner similar to Karp, and to test the comparability of results generated in C3H mice with those established in BALB mice. Consequently, suspensions of Karp, Kato, and Gilliam were exposed to gamma radiation doses in the range of 0-300 Krad and MLD₅₀ titrations were performed simultaneously at each dose in both mouse strains. The individual titers of the Karp and Kato suspensions were almost identical in BALB and C3H mice. Regression analyses (Table 1) indicated that the radiation dose-lethality curves for the rickettsial strains were quite similar and unaffected by choice of mouse model. The generation of complete data for Gilliam was precluded by the natural resistance of BALB mice to lethal infection with this strain of R tsutsugamushi (8). Corresponding results among mouse and rickettsial strain combinations also were observed in the PD₅₀ values obtained with 300 Krad gamma-irradiated immunogens (Table 2).

Protective doses for homologous strain challenge were quite similar for Karp immunogen titrated in either mouse strain, for Kato immunogen in BALB mice, and for Gilliam immunogen in C3H mice. However, a PD₅₀ for Kato in C3H mice could not be calculated. The highest concentration of Kato immunogen used failed to protect the animals from subsequent challenge. This apparent inability of C3H mice to develop protective immunity to Kato challenge was reproducible, but disturbing. Nevertheless, other values were comparable for the 2 mouse strains, and we felt that the benefit derived from broadened challenge studies warranted further use of C3H mice.

Table 1. Regression analysis of radiation dose-lethality curves of Karp, Kato and Gilliam in BALB and C3H mice.

Strain of R <u>tsutsu-</u> <u>gamushi</u>	Mouse strain	Log ₁₀ MLD ₅₀ of unirradiated suspension/g of yolk sac	100% lethal gamma dose (Krad) ^a	Slope (log ₁₀ MLD ₅₀ /g of yolk sac/Krad)	Correlation coefficient
Karp	BALB	-8.7	180	5.0 x 10 ²	0.98
	C3H	-8.8	170	5.2 x 10 ²	1.00
Kato	BALB	-8.6	190	4.4 x 10 ²	1.00
	C3H	-8.5	190	4.4 x 10 ²	1.00
Gilliam	C3H	-9.4	180	5.2 x 10 ²	1.00

^a Point at which calculations indicate there will be ≤ 1 lethal rickettsia/g of irradiated yolk sac.

Table 2. Immunogenicity of 300 Krad gamma-irradiated suspensions of R tsutsugamushi.

<u>R tsutsugamushi</u> immunogen strain	Protective dose of immunogen ^a BALBb	C3Hb
Karp	5.4 ± 0.3 ^c	5.5 ± 0.3
Kato	5.7 ± 0.4	≥ 8.2
Gilliam	ND ^d	6.3 ± 0.4

^a Expressed as log₁₀ PD₅₀, the number of MLD₅₀ units of immunogen required to protect 50% of vaccinated mice against a 10³ MLD₅₀ homologous strain challenge.

^b Mouse strain.

^c Value ± standard deviation.

^d ND = not determined.

Protection with single-strain immunogens. The use of C3H mice provided an opportunity to examine a matrix of homologous and heterologous protective capacities with immunogens prepared from R tsutsugamushi strains Karp, Gilliam, Kato, Kostival, and Buie. Animals were vaccinated using the standard 3-injection regimen, and were subdivided on day 31 for homologous and heterologous challenges. The results of the titrations, conducted in vaccines and controls, are expressed as immunity indices in Table 3. All of the immunogen preparations, including the Kato immunogen, were effective in protecting the animals against Karp and Gilliam challenge. Levels of cross protection indicated considerable antigenic similarity between Gilliam and Kostival and, to a lesser extent, between Karp and Buie. Further, Kostival and Buie appeared to be the most omnipotent of the immunogen strains tested, providing protection against all challenge strains except Kato. None of the immunogen strains examined, including Kato, provided detectable protection against Kato challenge, although a similar experiment employing irradiated Kato to vaccinate BALB mice yielded immunity indices of 6.4 ± 0.2 after Kato challenge and 5.2 ± 0.3 after Karp challenge.

Protection with three-strain combinations of immunogens. Three combinations of strains and 2 methods of effecting the combination within the standard vaccination regimen were examined. The combinations tested were: Karp-Gilliam-Kato (KGKt), Karp-Kostival-Kato (KKosKt), and Buie-Kostival-Kato (BKosKt). Vaccination was accomplished either by sequential administration of single-strain immunogens on days 0, 5, and 10 or by injection of a trivalent mixture on each of these days. The total mass of immunogen injected was similar regardless of the method of combination of strains. As in previous experiments, vaccinated animals were subdivided and challenged on day 31. Immunity indices for challenge with each strain and composite immunity indices for each strain combination are shown in Table 4. Individual immunity indices indicate that use of 3 immunogen strains for vaccination, regardless of strains combined or method of combination employed, resulted in detectable levels of protection against Kato and moderate to excellent protection against challenge with the other 4 strains. For each combination, composite immunity indices achieved with injection of the trivalent immunogen mixture were higher than those obtained by sequential injection of the single-strain suspensions. These differences in composite values were primarily due to the protection achieved against Kato challenge, where individual immunity indices indicate the protection levels increased 100 to 1000-fold with use of trivalent immunogens instead of sequential injection. Finally, composite immunity indices for the 3 trivalent immunogens indicate that the protective efficacies of KGKt and BKosKt

Table 3. Protection of C3H mice vaccinated with monovalent gamma-irradiated scrub typhus immunogens^a.

Immunogen strain	Total immunogen mass injected	Immunity index ^c				
		Karp ^d	Buie	Kato	Kostival	Gilliam
Karp	4 x 10 ⁸	6.1 ± 0.6	1.8 ± 0.5	≤ 1.2	≤ 0.6	3.2 ± 0.4
Buie	1 x 10 ⁸	5.0 ± 0.5	3.0 ± 0.4	≤ 1.0	1.4 ± 0.4	3.4 ± 0.4
Kato	2 x 10 ⁸	4.7 ± 0.5	≤ 0.8	≤ 1.0	≤ 0.7	3.8 ± 0.5
Kostival	2 x 10 ⁸	5.0 ± 0.5	1.6 ± 0.4	≤ 1.2	6.4 ± 0.4	6.0 ± 0.4
Gilliam	2 x 10 ⁸	3.0 ± 0.4	≤ 0.6	≤ 1.6	5.7 ± 0.4	5.8 ± 0.2

^a Mice were vaccinated with 3 i.p. injections of 300 Krad gamma-irradiated immunogen given at 5 day intervals and were challenged on day 31.

^b Expressed as MLD₅₀ units based on titration before irradiation.

^c Immunity index = log₁₀ MLD₅₀ in vaccinated mice - log₁₀ MLD₅₀ in control mice.

^d Challenge strain.

Table 4. Protection of C3H mice vaccinated with three-strain immunogen combinations^a.

Method of combining immunogen strains	Immunogen strain combination	Total immunogen mass injected ^b	Karp ^d	Individual challenge immunity index ^c			Composite immunity index ^c
				Buie	Kato	Gilliam	
Injection of one monovalent immunogen each day ^e	KGKt ^f	6 x 10 ⁸	5.6 ± 0.5	3.6 ± 0.5	1.6 ± 0.5	2.8 ± 0.5	4.6 ± 0.4
	KKosKt	8 x 10 ⁸	5.6 ± 0.3	3.9 ± 0.6	1.0 ± 0.5	5.4 ± 0.2	6.0 ± 0.3
	BKosKt	4 x 10 ⁸	6.6 ± 0.4	4.8 ± 0.4	2.0 ± 0.4	5.4 ± 0.4	5.6 ± 0.3
Injection of trivalent immunogen each day ^e	KGKt	5 x 10 ⁸	6.8 ± 0.4	4.5 ± 0.5	4.6 ± 0.4	6.2 ± 0.2	6.2 ± 0.3
	KKosKt	8 x 10 ⁸	5.6 ± 0.4	3.4 ± 0.6	3.8 ± 0.6	5.4 ± 0.2	6.0 ± 0.3
	BKosKt	4 x 10 ⁸	6.4 ± 0.4	5.2 ± 0.4	4.2 ± 0.4	6.4 ± 0.4	6.4 ± 0.3

^a Mice were vaccinated according to the standard regimen and challenged on day 31.

^b Expressed in MLD₅₀ units based on titration before irradiation.

^c Immunity index = log₁₀ MLD₅₀ in vaccinated mice - log₁₀ MLD₅₀ in control mice.

^d Challenge strain.

^e Sequential injection was administered in order of presentation (for KGKt, Karp on day 0, Gilliam on day 5, and Kato on day 10). Trivalent immunogens were prepared by mixing thawed monovalent immunogens in equal volumes just prior to injection.

^f KGKt, Karp-Gilliam-Kato; KKosKt, Karp-Kostival-Kato; BKosKt, Buie-Kostival-Kato.

were similar, and significantly higher, than that of KKosKt.

Duration of immunity after KGKt vaccination. Large groups of C3H and BALB mice were vaccinated with trivalent KGKt immunogen and challenged at intervals of 3 months over a period of 1 year (Table 5). With the exception of an unusually low value for Kato protection at 3 months, which may have been related to previously identified problems with Kato-C3H interactions, the levels of protection maintained by C3H mice over the first 6 months were relatively constant. The animals resisted challenge with over 10^5 MLD₅₀ of Karp, Kostival, and Gilliam; 10^4 MLD₅₀ of Buie; and 10^3 MLD₅₀ of Kato. By 9 months, a 100-fold reduction in protection levels was observed for Karp, Buie, and Kato, although levels of resistance to Kostival and Gilliam were undiminished. No further decline in resistance was observed for Gilliam, Karp, and Buie at 12 months, but immunity to Kato challenge could not be detected at that time. Conversely, BALB mice remained immune to high levels of Kato challenge throughout the study, evidencing a gradual decline from immunity to a 10^6 MLD₅₀ challenge at 3 months to protection against 10^3 MLD₅₀ of Kato at 12 months.

Duration of immunity after sublethal infection. The durability of infection-induced immunity to heterologous challenge was studied in BALB mice that had sustained a sublethal infection with 100 MLD₅₀ of Gilliam. Although a few random deaths were observed after each challenge, the mortality data (Table 6) indicated that the vast majority of mice were protected for a year against Karp strain challenge doses exceeding 10^7 MLD₅₀. Meaningful immunity indices could not be calculated because most mice survived the largest challenge doses employed.

This study indicated that the conditions previously established for inactivation of R tsutsugamushi strain Karp by gamma-radiation (6) generally are applicable to other strains of scrub typhus rickettsiae. Use of these immunogens as single-strains or multi-strain combinations with broad spectrum challenge delineated unexpected relationships among the rickettsial strains and revealed synergistic effects operative during multi-strain immunization.

All single-strain immunogens elicited high levels of protection against Karp and Gilliam. This was interesting, because previous serologic studies with these 2 strains, employing complement fixation tests (4,9) and rickettsial-neutralization tests (10,14), had indicated that they were antigenically distinct, although a minor complement-fixing antigen common to both strains had been detected (4).

Table 5. Duration of protection in mice vaccinated with KGKt trivalent gamma-irradiated scrub typhus immunogen^a.

Mouse strain	Challenge strain	Immunity index ^b			
		3 ^c	6	9	12
C3H	Karp	5.6 ± 0.4 ^d	5.8 ± 0.5	3.8 ± 0.5	4.0 ± 0.7
	Buie	4.2 ± 0.6	4.2 ± 0.6	1.6 ± 0.5	1.4 ± 0.6
	Kostival	5.2 ± 0.3	5.6 ± 0.4	6.3 ± 0.4	N.D. ^e
	Gilliam	5.6 ± 0.5	5.6 ± 0.3	5.6 ± .5	5.1 ± 0.6
BALB	Kato	1.8 ± 0.5	3.6 ± 0.5	1.7 ± 0.6	0.4 ± 0.3
	Kato	6.0 ± 0.4	4.8 ± 0.3	5.8 ± 0.5	3.0 ± 0.5

^a Mice were vaccinated by the standard regimen with 5.8×10^8 MLD₅₀ units of Karp-Gilliam-Kato immunogen/mouse.

^b Immunity index = \log_{10} MLD₅₀ in vaccinated - \log_{10} MLD₅₀ in control mice.

^c Months after immunization.

^d Value ± standard deviation.

^e Not done.

Table 6. Duration of immunity to Karp challenge in BALB mice convalescent from a sublethal Gilliam infection^a.

Challenge month	No. of survivors/No. of immunized mice challenged				
	10 ⁴ b	1M	100K	1K	100
1	5/5	5/5	5/5	4/5	
6	5/5	5/5	5/5	5/5	5/5
9	5/5	4/5	4/5	5/5	5/5
12	4/5	5/5	4/5	4/5	5/5

^a Mice were infected by i.p. injection of 100 MLD₅₀ of Gilliam.

^b MLD₅₀ Karp strain challenge.

The Buie and Kostival immunogens appeared to be broadly reactive eliciting moderate to high levels of protection against Karp, Gilliam, and homologous strain challenge while showing low, but detectable levels of protection against each other. These observations agree with previous reports of broad reactivity for rabbit anti-Buie serum in cross-neutralization tests (10) and for formalinized Kostival immunogen in cross-vaccination studies (5).

As predicted by our preliminary lethality and immunogenicity studies, none of the immunogens, including the irradiated Kato suspension, protected the C3H mice against Kato challenge. However, the same Kato suspension elicited high levels of protection against Karp and Gilliam in C3H mice and against Kato in BALB mice, indicating that the limitation of the C3H model was restricted to use of Kato challenge. We can offer no explanation for the phenomenon at this time, although it does illustrate the importance of ascertaining the limitations of an animal model.

Previous studies with mixed, formalinized immunogens (5) indicated that a reduction in protection against each challenge strain occurred, and was proportional to the dilution effect achieved by mixing the immunogens. Nevertheless, the demonstrable superiority of gamma-irradiated immunogens (6) and the pronounced cross-protection observed against Karp and Gilliam with the single-strain immunogens (Table 3) encouraged us to attempt the use of multi-strain combinations in this study. We chose to use 3 immunogen strains because the 3-injection regimen allowed combination of Karp, Gilliam, and Kato by sequential injection in single-strain form on successive vaccination days, as well as injection of a pre-mixed suspension. We also examined 2 other combinations employing the Buie and Kostival strains, since results of the single-strain study suggested they were slightly more immunogenic than Karp or Gilliam. The principal result of using any of the multi-strain combinations was a vast improvement in the breadth and level of protection over that afforded mice vaccinated with any of the single-strain immunogens. For the first time, vaccinated C3H mice resisted significant levels of Kato challenge. This was particularly striking after immunization with the trivalent mixtures, and could not be attributed to increased immunogen mass as less Kato was present in the KGKt mixture than had been employed in the single-strain vaccination studies. Protection against Buie challenge also increased when immunogen combinations were used, with the immunity index rising from 3.0 after single-strain Buie immunization to 4.5 with the KGKt trivalent mixture. The immunity indices achieved for Karp, Kostival, and Gilliam challenges after vaccination with trivalent mixtures were generally similar to those

attained against homologous strain challenge with single-strain immunogens.

In order to fully evaluate the efficacy of the trivalent immunogens in mice, it was necessary to examine the duration of protective immunity. In a previous study (7) we reported that BALB mice vaccinated with Karp immunogen retained immunity to challenge with 10^4 MLD₅₀ of Karp for 9 months, but became susceptible to challenge with low levels of Kato after only 3 months. In contrast, mice convalescent from sublethal R tsutsugamushi infection have been shown to resist intraperitoneal inoculation of 10^4 MLD₅₀ of homologous strain organisms for at least 610 days (15), and in this study the majority of BALB mice convalescent from sublethal Gilliam infection resisted heterologous strain challenge with 10^6 MLD₅₀ of Karp for 1 year. In comparison, the trivalent KGKt gamma-irradiated immunogen elicited reduced, but significant levels of protection. C3H mice resisted challenge with 10^3 MLD₅₀ of Karp and 10^4 MLD₅₀ of Gilliam for 12 months, and it is likely that a similar level of protection was maintained against Kostival challenge. Immunity indices for Kato and Buie challenges remained at acceptable levels in C3H mice for only 6 months, although the vaccinated BALB mice continued to resist challenge with 10^3 MLD₅₀ of Kato for 12 months.

These vagaries in mouse response, most evident in the C3H-Kato interaction, emphasize the effect of the animal model on testing of scrub typhus immunogens. In this study, use of either BALB or C3H mice alone would have presented a biased view of the effectiveness of irradiated rickettsiae. Thus, sole use of BALB mice would have limited the number of challenge strains employed and prevented us from observing the synergistic effect produced by vaccination with polyvalent immunogens. Conversely, use of only C3H mice would have led to the conclusion that radiation was totally unsuitable for the preparation of Kato immunogens. Interpretation of data from both strains of mice revealed that the KGKt trivalent immunogen induced protective immunity to broad spectrum challenge for 6 months. Studies are currently in progress to prepare trivalent scrub typhus immunogen from rickettsiae grown in appropriate cell culture for testing in sub-human primates.

2. Gamma-irradiated scrub typhus immunogens: analysis for residual, replicating rickettsiae.

In a recent study (6), BALB/cDub (BALB/c) mice vaccinated with gamma-irradiated, Karp strain scrub typhus immunogens resisted challenge with 200 50% mouse lethal doses (MLD₅₀) of untreated, virulent homologous strain rickettsiae that had been introduced artificially into the immunogen inoculum just prior to vaccination. Additional experiments conducted in that study indicated that the phenomenon, termed "radiation masking", was attributable to early development of strain-specific immunity induced by the massive dose of inactivated rickettsiae introduced in the initial injection of immunogen. Although the vaccinated mice showed no signs of active infection after receiving immunogen containing 2 to 200 MLD₅₀ of virulent Karp rickettsiae, transfer of homogenized spleen cells from these mice caused lethal infections in normal syngeneic recipients. Thus, the donors had sustained and survived infections that would have been lethal for unprotected mice. Similar results were obtained when the experiments were repeated using BALB/c, C3H/HeDub (C3H), and outbred WRC:(ICR) mice (G. H. G. Eisenberg, Jr. and J. V. Osterman, Unpublished Data). The results suggested that previous assumptions made about infectivity and safety of irradiated suspensions of R. tsutsugamushi may not be valid. Survival of vaccinated mice is not sufficient evidence to prove rickettsial inactivation is complete and potentially virulent organisms are absent from an inoculum.

Transfer of spleen cell homogenates from vaccinated to unvaccinated mice represents another method, in addition to plaque titration in tissue culture (16), that can be used to evaluate gamma-irradiated rickettsial suspensions for the presence of "masked" residual, replicating rickettsiae. The purpose of this study was to compare the efficacy of spleen cell homogenate transfer and plaque titration in detecting viable rickettsiae in irradiated suspensions of Karp, Gilliam and Kato.

Regression analysis of gamma-irradiation dose-lethality curves for each of the three rickettsial suspensions used indicated that the minimum radiation dose required for total inactivation of the rickettsiae was 170 Krad for Karp, 180 Krad for Gilliam, and 190 Krad for Kato. Aliquots of each rickettsial strain preparation were exposed to irradiation doses of 150, 200, 250, and 300 Krad (6), and stored at -70C. Plaque titrations were performed as previously described (16). Each of 15 culture dishes of irradiated L-929 cells was inoculated with 0.2 ml of the appropriate rickettsial suspension diluted 1:4 in brain heart infusion broth. Female C3H mice (Flow Laboratories, Inc.,

Dublin, VA), 18 to 22 g, were used for in vivo testing. Irradiated rickettsial suspensions were given by intraperitoneal injection in a standard volume of 0.2 ml. Donor mice were killed for spleen cell harvest 24 days after injection of the irradiated 20% yolk sac suspension. Homogenates were prepared from the pooled, unfractionated spleen cell suspensions (17), and 1 mouse spleen-equivalent of homogenate was injected intraperitoneally into each recipient mouse. Recipients were observed for deaths occurring 6 to 28 days after transfer. Surviving recipients were challenged with 1,000 MLD₅₀ of fully virulent, homologous strain rickettsiae and observed for an additional 28 days.

The gamma irradiation dose of 150 Krad was lower than the calculated minimum inactivation dose for any of the 3 scrub typhus strains. This exposure dose was selected to permit evaluation of the sensitivity of the 2 analytical methods to detect minimum numbers of viable, active rickettsiae present in an immunogen preparation that had been inadequately irradiated.

Results for the two analytical procedures are shown (Table 7). Residual, replicating rickettsiae were detected by both procedures in the Gilliam and Kato suspensions irradiated at 150 Krad, whereas "masked" rickettsiae in the 150 Krad-treated Karp suspension were detected only by the in vivo procedure. The estimated concentration of viable rickettsiae in the Gilliam and Kato suspensions was 10 plaque forming units (pfu) per ml.

Plaque assay for rickettsiae requires the cultures to be incubated for 19 days, with a feeder overlay being applied on day 9 (16). By contrast, mouse inoculation analysis takes a longer time to complete, but involves fewer manipulations. Thus, when masking of organisms occurs, as illustrated by the Karp and Gilliam preparations, the total time consumed will approach 80 days. Homogenization of the spleen cells prior to transfer normally resulted in the release of viable rickettsiae that induced lethal infections in the recipients, as illustrated by the Gilliam suspension treated at 150 Krad. With the Karp preparation, however, a second non-lethal infection occurred following transfer of the spleen cell homogenate. This inapparent infection was demonstrated only when the recipients survived a subsequent challenge with 1,000 MLD₅₀ of homologous rickettsiae, a dose known to be lethal for unprotected mice. Kato immunogen does not protect C3H mice against Kato challenge (G. H. H. Eisenberg, Jr. and J. V. Osterman, Manuscript in Press); therefore, the presence of residual, replicating rickettsiae is readily demonstrable upon initial injection into C3H mice. The absence of "masked" Kato rickettsiae allowed the residual organisms to be detected in the same time interval as for plaque assay.

Table 7. Analysis of irradiated suspensions of R tsutaugamushi for residual replicating organisms.

Strain	Krad gamma irradiation	No. plaques/no. inoculated plates examined ^a	No. survivors/ no. mice injected ^b	No. survivors/ no. transfer recipients ^c	No. survivors/ no. challenged recipient survivors ^d
Karp	150	0/13	10/10	5/5	4/5
	200	0/14	10/10	5/5	0/5
	250	0/5	10/10	5/5	0/5
	300	0/6	10/10	5/5	0/5
Gilliam	150	2/6	10/10	4/5	0/4
	200	0/7	10/10	5/5	0/5
	250	0/6	10/10	5/5	0/5
	300	0/15	9/9	5/5	0/5
Kato	150	2/4	0/6	---	---
	200	0/7	6/6	5/5	0/5
	250	0/9	7/7	5/5	0/5
	300	0/2	10/10	4/5 ^e	0/4

^a Number less than 15 indicates loss of cultures due to laboratory accident.

^b Each mouse given 0.2 ml of irradiated 20% yolk sac suspension by intraperitoneal injection.

^c Recipients received 0.2 ml intraperitoneally of a suspension containing one mouse equivalent of homogenized spleen cells harvested from mice surviving 24 days intraperitoneal injection of irradiated yolk sac suspension.

^d Surviving spleen cell recipients were challenged with 1,000 MLD₅₀ of homologous strain rickettsiae given intraperitoneally at 28 days after transfer.

^e Recipient died 24 days after injection without evidencing previous signs of disease.

As expected from the calculated minimum radiation doses, residual organisms only were detected in the 3 suspensions exposed to 150 Krad. In previous studies (6,7), however, the active rickettsiae retained the ability to induce lethal infection in unprotected mice. It suffices to note, for the purpose of this study, that the transfer of spleen cell macerate followed by challenge inoculation was capable of detecting the presence of infectious organisms even if they exhibited altered virulence. This phenomenon of altered virulence needs and warrants further investigation to ascertain whether it is a stable deviation and whether it occurs sufficiently often to be of value in vaccine production.

Neither the plaque or the mouse assay indicated the presence of viable organisms in suspensions exposed to 200, 250, or 300 Krad. The one recipient mouse that died on day 24 after injection of spleen macerate from donors vaccinated with 300 Krad Kato immunogen undoubtedly died of causes unrelated to rickettsial infection. This mouse did not exhibit previous signs of illness, whereas unprotected mice normally show clinical signs two to three days prior to death and most deaths occur by day 17 after inoculation. Further, "radiation masking" is non-existent in the Kato-C3H mouse model (18) and the findings were negative for residual organisms in the Kato suspensions exposed to 200 and 250 Krad.

The combination of plaque assay and spleen macerate in vivo analysis was of sufficient sensitivity to detect miniscule numbers of residual, replicating rickettsiae in irradiated scrub typhus immunogens. Although the time required for completion of analysis was increased by approximately four-fold, the in vivo procedure exhibited the greatest degree of sensitivity. Using the in vivo procedure as designed, both virulent and avirulent residual rickettsiae were detected.

3. Adjuvant effect of Coxiella burneti vaccine and soluble phase I Coxiella burneti antigen on immune response to gamma-irradiated scrub typhus immunogens.

Gamma-irradiated scrub typhus immunogens have been shown to induce immune responses in mice that would protect against high homologous challenge levels (6,7,18,19). Broad-spectrum protection was achieved by using a combination of 3 strains (18). The trivalent Karp-Gilliam-Kato immunogen elicited protection levels lasting a minimum of 12 months.

Agents capable of specifically modifying host immune responses in a beneficial manner against infectious agents are being sought and studied because they may provide an additional modality for the

prevention and treatment of disease. A large number of agents, microbial and chemical, have been shown to be capable of modulating the host immune response. We decided to investigate the adjuvant potential of Coxiella burneti to enhance nonspecifically the immune response to gamma-irradiated scrub typhus immunogens. A commercially produced extract of C burneti for use as antigen in the complement fixation test afforded protection to mice against subsequent infection with Babesia spp and Plasmodium spp (20), and the lipopolysaccharide component, as well as killed intact, C burneti was reported to induce activation of guinea pig macrophages (21). Further, immunization of military troops against these organisms would be desirable in endemic areas of the world.

Mice. Female BALB/c mice, 18 to 22 g, obtained from Flow Laboratories, Dublin, VA, were used.

Rickettsiae. The Karp strain (53rd egg passage) and Kato strain (200th egg passage) of Rickettsia tsutsugamushi were propagated, stored, and quantified by methods reported previously (11). Suspensions used had a titer $\geq 1 \times 10^8$ MLD₅₀/g of yolk sac.

Rickettsial immunogen. Radiation-inactivated Karp immunogens were prepared by exposing 20% yolk sac suspensions of the Karp strain to 300 Krad gamma radiation as described previously (6).

Coxiella burneti immunogens. Q fever vaccine (phase I, inactivated, dried, code No. NDBR 105) and soluble phase I C burneti antigen (aqueous) were obtained from Dr. R. Wachter, USAMRIID, Ft. Detrick, MD. The Q fever vaccine (NDBR) was diluted with 0.85% NaCl solution to contain 2 ug of mass/0.2 ml and the soluble phase I antigen (Sol Ph I) was diluted to 4 ug of protein/0.2 ml.

Vaccination and challenge. All injections of rickettsiae and rickettsial antigens were given intraperitoneally (ip) and injections of C burneti antigens were administered subcutaneously (sc), each in a standard volume of 0.2 ml. On day -4, one group of mice was given NDBR (2ug/mouse) and a second group was given Sol Ph I (4ug/mouse). Third and fourth groups of mice received NDBR and Sol Ph I, respectively, on day 0. Simultaneously on day 0 mice in each group were vaccinated with Karp immunogen using dilutions in 10-fold increments calculated to encompass dose ranges between 10^3 and 10^8 MLD₅₀ (expressed as MLD₅₀ units based on titration before irradiation). Challenge doses (1000 LD 50) of Karp (homologous strain) or Kato (heterologous strain) were injected 30 days

after the vaccination regimen. On the day of challenge, standard suspensions of the challenge inocula were titrated in normal control mice. Deaths occurring 6 to 28 days postchallenge were quantified. The \log_{10} MLD₅₀ and standard deviation were calculated by the Spearman-Kärber method (12). The 50% protective dose (PD₅₀) was expressed in MLD₅₀ units by calculating the difference between the \log_{10} of the 50% protective dilution and the \log_{10} of the MLD₅₀ of the unirradiated suspension.

Results observed in the PD₅₀ values are shown in Table 8. Protective doses for homologous and heterologous challenge were quite similar for the immunogens Karp, Karp simultaneously with NDBR or Sol Ph I, and Karp after Sol Ph I. When Karp immunogen was administered 4 days after NDBR there appeared to be a slight potentiation of the immune response; however, the degree of enhancement observed was not statistically significant.

Potentiation of the immune response to a given antigen in the presence of adjuvants has been reported for many biological systems (22), often resulting in a multi-fold increase in immune resistance. The mechanisms of action of adjuvants on stimulating the immune responses currently has not been defined, and most likely involves one or more of several modes. The practical use of adjuvants depends on definition of the relationship of adjuvant to potential antigen.

In this study, significant enhancement was not observed of the immune response to scrub typhus immunogen when C burneti immunogens were used as adjuvants. Recent studies in this laboratory (18) demonstrated the development of broad-spectrum protection to scrub typhus following administration of trivalent gamma-irradiated R tsutsugamushi immunogen. Additional studies will elucidate whether the C burneti NDBR vaccine will exert a potentiating effect when given prior to this trivalent mixture.

4. Nonspecific resistance of mice to infection with scrub typhus rickettsiae.

Mouse peritoneal macrophages activated in vivo during scrub typhus infection, or in vitro with lymphokines, were nonspecifically tumoricidal and refractory to Rickettsia tsutsugamushi infection in vitro. Analysis of the rickettsiacidal activity of activated macrophages suggested 2 distinct mechanisms: (1) an immediate decrease in infectivity of rickettsiae for macrophages and (2) a subsequent, 24 hr, increase in intracellular killing of the rickettsiae (Table 9).

Table 8. Adjuvant effect of Coxiella bruneti immunogens on immunity induced by Karp strain Rickettsia tsutsugamushi gamma-irradiated immunogen.

Immunogen	Protective Dose of Immunogen (\log_{10} PD ₅₀) ^a	
	Karp ^b	Kato
Karp	5.8 \pm 0.4 ^e	6.9 \pm 0.4
Karp + NDBR ^c	5.8 \pm 0.4	6.7 \pm 0.4
Karp + Sol Ph I ^c	5.8 \pm 0.4	6.7 \pm 0.4
Karp After NDBR ^d	4.8 \pm 0.4	6.0 \pm 0.4
Karp After Sol Ph I ^d	5.9 \pm 0.2	7.4 \pm 0.3

^a \log_{10} PD₅₀ = number of MLD50 units of immunogen required to protect 50% of vaccinated mice against a 10³ MLD50 challenge.

^b Challenge strain of R tsutsugamushi.

^c C bruneti immunogens administered simultaneously with Karp strain R tsutsugamushi immunogen.

^d C bruneti immunogens administered 4 days prior to giving Karp strain R tsutsugamushi immunogen.

^e Value \pm standard deviation.

Early host response to experimental scrub typhus infection, prior to the development of humoral immunity, involves macrophages and T lymphocytes. In order to study the mechanism(s) of rickettsiae-macrophage interaction during this time, nonspecific resistance to scrub typhus was induced by intraperitoneal injection of Mycobacterium bovis, strain BCG. Susceptible mice were protected against various challenge doses of the Gilliam and Karp strains of R tsutsugamushi (Table 10). Complete protection was not achieved with prior BCG treatment against challenge with a third major antigenic strain, Kato; however, survival of these mice was prolonged 4 to 6 days.

Activated macrophages obtained from BCG-infected mice at the time of rickettsial challenge demonstrated both rickettsiacidal and tumoricidal activity in vitro (Table 11). The ability of macrophages from BCG-infected mice to kill rickettsiae was similar to that observed with macrophages obtained from mice with regressing scrub typhus infection. This rickettsiacidal activity of macrophages from BCG-infected mice clearly demonstrated the nonspecific nature of the rickettsiae-macrophage interaction.

The nonspecific resistance of mice to R tsutsugamushi infection following treatment with BCG was not due solely to inflammatory responses by the host. Sterile irritants (phosphate-buffered saline, latex beads, and thioglycollate) did not induce protection against rickettsial infection (Table 12). Macrophages obtained from sterile irritant-stimulated mice at the time of rickettsial challenge did not kill rickettsiae unless the macrophages subsequently had been activated in vitro by lymphokines.

Nonspecific resistance to rickettsial infection depended upon the presence of activated macrophages for a critical time following challenge. Acute immune responses in mice initiated by intraperitoneal inoculation of PPD, intraperitoneal inoculation of PPD 10 days after infection intradermally with BCG, or intraperitoneal inoculation with concanavalin A did not protect against challenge with either Gilliam or Karp strains of R tsutsugamushi (Table 13). Protection was afforded to those mice exhibiting chronic immune responses stimulated by intraperitoneal infection with BCG or nonlethal doses of Gilliam scrub typhus rickettsiae. However, macrophages harvested at the time of challenge from mice treated with macrophage activating agents were nonspecifically rickettsiacidal and tumoricidal (Table 14).

Table 9. Rickettsiacidal activity of resident and activated macrophages by R tsutsugamushi, strain Gilliam.

Treatment	% Rickettsiacidal Activity		% Tumor Cytotoxicity
	Immediate	24 hr	
Macrophages from uninfected mice:			
Medium	0	0	7
Lymphokines	40	75	53
Macrophages from rickettsia-infected mice:			
Medium	42	88	70

Table 10. Nonspecific resistance to R tsutsugamushi induced by inoculation of Mycobacterium bovis, strain BCG.

Rickettsial strain	Challenge Dose (log ₁₀ MLD ₅₀)	% Survival	
		Controls	BCG Infected ^a
Gilliam	4	0	75
	3	0	100
	2	0	100
	1	0	100
Karp	4	0	75
	3	0	50
	2	0	75
	1	0	75

^a Mice were challenged with R tsutsugamushi 10 days after inoculation with M bovis, strain BCG.

Table 11. Rickettsiacidal activity of macrophages in vitro following treatment of mice with M bovis, strain BCG.

Treatment in vivo	Lymphokines in vitro	% Rickettsiacidal Activity		% Tumor Cytotoxicity
		Immediate	24 hr	
None	-	0	0	8
	+	40	75	47
BCG	-	42	86	75
R tsutsugamushi strain Gilliam	-	53	86	79

Table 12. Rickettsiacidal activity in vitro of macrophages induced by sterile irritants.

Treatment in vivo	Lymphokines in vitro	% Rickettsiacidal Activity		% Tumor Cytotoxicity
		Immediate	24 hr	
PBS	-	0	0	10
	+	50	80	44
Latex Beads	-	0	0	11
	+	46	60	52
Thioglycollate	-	0	0	8
	+	42	68	43
None	-	0	0	10
	+	40	78	41

Table 13. Survival of rickettsial challenge following inoculation of macrophages activating agents.

Treatment in vivo	% Survival 1000 MLD ₅₀ Challenge	
	Gilliam	Karp
Intradermal BCG	0	0
Intradermal BCG + intraperitoneal PPD	0	0
Intraperitoneal PPD	0	0
Intraperitoneal concanavalin A	0	0
Intraperitoneal BCG	100	75
Intraperitoneal Gilliam (nonlethal dose)	100	100
Untreated	0	0

Table 14. In vivo activation of macrophages for killing of *Rickettsia tsutsugamushi*.

Treatment in vivo	% Rickettsiacidal Activity		% Tumor Cytotoxicity
	Immediate	24 hr	
Intradermal BCG	0	6	14
Intradermal BCG + intraperitoneal PPD	36	74	50
Intraperitoneal PPD	0	0	17
Intraperitoneal concanavalin A	0	50	48
Intraperitoneal BCG	46	72	57
Intraperitoneal Gilliam (nonlethal dose)	48	80	72
Untreated	0	0	12

5. Macrophage activation in vitro for killing of *Rickettsia tsutsugamushi*.

The pathogenesis of rickettsial infections in susceptible animals is complex and poorly understood. Wide variation exists not only in the interaction of different rickettsial species with a single host, but in the response of different hosts to a particular strain of rickettsia (23,24). A role for cellular immunity in resistance to rickettsial diseases was suggested by both experimental and clinical studies that documented the development of cell-mediated immune responses following infection (25,26). The precise nature of cellular immunity during rickettsial infection is still largely unknown. Several lines of evidence suggested that the macrophage, as in infections with other obligate intracellular organisms (27, 28,29), may be a major effector cell in antirickettsial immunity. Rickettsiae preferentially infect endothelial cells of small blood vessels, and macrophages with intracellular organisms are regularly detected in the perivascular inflammatory infiltrates (E. Ewing, personal communication). Studies with *Rickettsia mooseri* and *Coxiella burnetii* have described in vitro model systems in which macrophages treated with supernatants of mitogen-stimulated leukocyte cultures (30) or exposed to immune serum-treated rickettsiae (31,32) developed enhanced rickettsiacidal activity. An early and vigorous development of T cell immunity has been demonstrated during experimental scrub typhus infection, and protection could be passively transferred with T-cell enriched murine spleen cells (33). We have recently demonstrated a role for activated macrophages in resistance to *Rickettsia tsutsugamushi*, the etiologic agent of scrub typhus (34). Macrophage cultures activated in vitro with supernatants of antigen-stimulated spleen cells of rickettsiae-infected mice, or in vivo during nonlethal scrub typhus infection, were refractory to infection with *R. tsutsugamushi*, strain Gilliam, in vitro. We have further analyzed the interaction of soluble products from spleen cells of rickettsiae-infected mice and normal macrophages, and described both lymphokine concentration-dependence and the kinetics of macrophage activation for rickettsial killing. Lymphokine-treated macrophages exhibited potent anti-rickettsial activity in vitro which was the net result of at least 2 distinct mechanisms.

Animals. Adult female BALB/c mice, 18-22 g, were purchased from Flow Laboratories, Dublin, VA. C3H/HeN male mice were obtained from the Division of Research Sciences, National Institute of Health, Bethesda, MD.

Spleen cell culture supernatants. BALB/c mice were infected intraperitoneally (ip) with a sublethal inoculum (1000 plaque

forming units (PFU)) of R tsutsugamushi, strain Gilliam. Spleens were removed 21 days after infection, minced, and passed through 50 mesh stainless steel sieves into tissue culture medium RPMI 1640. Spleen cells were centrifuged at 500 x g for 10 min at 4 C, washed twice in medium, and resuspended to a concentration of 5×10^6 viable cells/ml. Twenty ml of spleen cell suspension with and without heat-killed rickettsiae (2×10^6 rickettsiae/ml) were incubated in 75 cm² plastic flasks for 30 hr at 37 C. Supernatant fluids from replicate cultures were pooled, centrifuged at 500 x g for 10 min at 4 C, and passed through a 0.2 μ m Nalgene filter. Aliquots were stored at 4 C until use. Lymphokine-containing culture supernatants retained their activity for 6 months or longer.

Peritoneal cells. Resident peritoneal cells (PC) from untreated BALB/c mice were collected after ip injection of 6 to 8 ml L-15 medium supplemented with 2% heat-inactivated fetal bovine serum (FBS), 50 μ g/ml of gentamicin, and 10 U/ml of sodium heparin. Peritoneal fluid was withdrawn through the ventral abdominal wall with a 20 gauge needle. Fluids from 3 to 10 mice were pooled, samples were removed for differential and total cell counts, and the remainder was centrifuged at 500 x g for 10 min at 4 C. Differential counts were made on Wright-stained cell smears prepared by cytocentrifugation. Washed PC suspensions were adjusted to 1×10^6 macrophages/ml in RPMI 1640 with 10% FBS. Macrophages were exposed to spleen cell culture supernatants as adherent PC monolayers. PC suspensions ($2.5 \times 10^6/2.5$ ml) were added to 35 mm culture wells with glass coverslips and incubated in dilutions of spleen cell supernatants for 4 hr. In certain experiments, unfractionated PC suspensions were treated with spleen cell supernatants (5×10^5 macrophages/0.5 ml) in polypropylene tubes.

Estimation of rickettsial growth in peritoneal macrophages. R tsutsugamushi, strain Gilliam, was stored, propagated, and quantified as previously described (16). Viable organisms, approximately 15 PFU/macrophage, were added to unfractionated or adherent PC cultures and incubated for 1 hr with rotation of culture plates or shaking of tubes every 10 min. Infected PC were washed twice to remove extracellular rickettsiae and incubated for an additional 0 to 24 hr at 34 C with CO₂ in moist air in medium or dilutions of spleen cell supernatants. The percentage of macrophages with intracellular rickettsiae was determined for adherent PC cultures by examination of Giemsa-stained cells on glass coverslips removed from culture wells. For unfractionated PC suspensions, percentage of infected macrophages was determined by examination of Giemsa-stained cell smears prepared by cytocentrifugation. Results were expressed as mean percent rickettsiae-free macrophages \pm standard error of the mean for 4 observations made on duplicate cultures.

The numbers of viable intracellular rickettsiae were estimated by titration of macrophage lysates for plaque formation in irradiated L-929 cell monolayers (16). Briefly, PC cultures with 1×10^6 macrophages/ml were treated with medium or 1/10 dilution of lymphokines for 4 hr. Cultures were washed and exposed to viable rickettsiae at a multiplicity of infection of about 15 PFU/macrophage for 1 hr. Infected cultures were washed, adjusted to equal macrophage concentrations and either lysed immediately by blending in a Sorvall blender with microadapter or incubated in medium or lymphokines for an additional 18 hr, then lysed. Lysates from equal numbers of macrophages were titrated in L-929 monolayers. Results are expressed as mean number of plaques \pm standard error of the mean in 3 to 6 L-929 monolayers for 3 separate dilutions of macrophage lysate.

Macrophage induced tumor cytotoxicity. Peritoneal exudate cells from C3H/HeN mice treated 18 to 24 hr previously with 1.0 ml of 0.02 M phosphate-buffered saline (PBS) were collected and cultured for 2 to 3 hr. Non-adherent PC were removed by repeated washing and the adherent PC incubated for 4 hr with dilutions of spleen cell supernatants. All cultures were washed and incubated with $^3\text{HTdR}$ -labeled mKSA TU-5 tumor cells (4×10^4 tumor cells/well) as previously described (35). Cytotoxicity was estimated by measurement of $^3\text{HTdR}$ release into culture supernatants, and expressed as mean cpm \pm standard error of the mean for triplicate cultures, and as percent total counts. Labeled tumor cell monolayers digested with 0.5% sodium dodecyl sulfate in water were used to estimate total counts.

Rickettsial growth in macrophage cultures. R tsutsugamushi, strain Gilliam, infects and replicates in monolayer cultures of resident peritoneal macrophages from BALB/c mice. Intracellular rickettsiae were evident in 20 to 30% of macrophages exposed to 15 PFU R tsutsugamushi/cell for 1 hr. Numbers of infected macrophages, as well as number of intracellular rickettsiae/macrophage, increased with time in culture. Recent experiments using unfractionated peritoneal cells and a range (3 to 30) of PFU rickettsiae/cell confirmed the earlier observations (Table 15).

Rickettsiacidal activity in macrophage monolayers treated with spleen cell culture supernatants. Rickettsiae-macrophage interactions were markedly affected by prior treatment of macrophage monolayers with culture supernatants of spleen cells from rickettsiae-infected mice (Table 16). Specifically, culture supernatants of spleen cells incubated with or without heat-inactivated rickettsiae were added for 4 hr to monolayers of adherent peritoneal cells (greater than 90% macrophages) from

Table 15. Growth of Rickettsia tsutsugamushi, strain Gilliam, in cultures of nonactivated macrophages from BALB/c mice.

Time	Percent macrophages infected	Average number rickettsiae/infected macrophage
1 hour	21 \pm 1 ^a	1.3 ^b
1 day	25 \pm 1	3.3
2 day	25 \pm 1	4.6
3 day	36 \pm 3	7.8
4 day	61 \pm 4	15.2

^a Value \pm standard error of the mean.

^b A minimum of 150 infected macrophages was examined.

Table 16. Rickettsial infection of normal BALB/c macrophage monolayers treated with supernatants of spleen cell cultures from infected mice and normal mice.

Treatment ^a	Reduction in Rickettsiae- infected Cells ^b		Tumor Cytotoxicity ^c	
	Immediate	24 Hr	CPM \pm SEM	% Total Counts
Spleen cell culture supernatant fluid from rickettsiae- infected mice	(+) 58%	58%	1480 \pm 110	39%
	(-) 16%	55%	670 \pm 0	12%
Spleen cell culture supernatant from normal mice	(+) 17%	7%	740 \pm 10	13%
	(-) 8%	0	710 \pm 30	14%
Medium (RPMI 1640)	(+) 10%	15%	740 \pm 20	12%
	(-) 0%	0%	740 \pm 0	13%
Total Counts: 5260 \pm 100				

^a Parentheses indicate whether spleen cells were incubated with or without heat-killed rickettsiae prior to harvesting supernatant.

^b Ratio of the difference between infected macrophages in treated and untreated cultures to infected macrophages in control cultures X 100 (%).

^c Release of ³H-thymidine from triplicate cultures of prelabeled target cells.

normal mice after which time the monolayers were washed and infected with viable rickettsiae for 1 hr. Percentage of macrophages with intracellular rickettsiae was determined by microscopic examination of stained cell smears immediately after infection and 24 hr later. Immediately after the 1 hr exposure to viable organisms, the percentage of macrophages free of intracellular rickettsiae was significantly increased (approximately twice that of medium-treated controls) in macrophage monolayers treated with soluble products (lymphokines) of spleen cells from infected mice incubated with heat-killed rickettsiae. Supernatants of spleen cells from control mice incubated with or without rickettsiae had little or no effect on the number of rickettsiae-infected macrophages. By 24 hr, an increase in rickettsiae-free macrophages was evident in monolayers treated with supernatants of spleen cells from infected mice incubated without rickettsiae. The tumoricidal activity also was measured as an index of macrophage activation (Table 16). There was complete correlation between the increase in rickettsiae-free macrophages and the development of tumoricidal activity in macrophage cultures treated with lymphokines from antigen-stimulated spleen cells of rickettsiae-infected mice. Supernatants of spleen cells from infected mice incubated without rickettsiae, while enhancing the number of macrophages resistant to rickettsial infection in 24 hr, did not activate macrophages for tumor cytotoxicity.

Rickettsial infection of macrophage cultures treated with lymphokines prior to infection. Macrophage cultures were treated with lymphokines for 4 hr, washed, and exposed to viable rickettsiae for 1 hr. Cultures were washed after infection and examined microscopically at 24 hr to determine percentage of infected macrophages. Rickettsiae-free macrophages were significantly increased in lymphokine-treated macrophage cultures. This increase was dependent upon lymphokine concentration, and significant activity persisted through a 1:10 dilution of active supernatants. Suppression of rickettsial growth in macrophages after lymphokine pretreatment was equally evident in both adherent PC monolayers (90 to 95% macrophages, Table 16) and in unfractionated PC suspensions (50 to 60% macrophages). Analysis of lymphokine effects in PC suspensions, however, avoided possible artifacts associated with removing adherent cells for sampling, or with changes in cell adherence during treatment, and was therefore, used for the remainder of these studies. Macrophages were treated with a 1:10 dilution of active supernatants for 0 to 24 hr. Treated macrophages were washed, exposed to viable rickettsiae for 1 hr, and examined immediately or 24 hr later for percentage of macrophages with intracellular rickettsiae. An increase in rickettsiae-free macrophages was evident with 1 hr lymphokine pretreatment, reached maximal levels after 6 to 12 hr, then

progressively decreased to control levels by 24 hr. This time course suggested that lymphokine effects on rickettsiae-macrophage interaction were rapid in onset, but relatively short-lived. Loss of lymphokine effects occurred gradually over 24 hr in culture, even in the continued presence of active lymphokine-rich supernatants. Comparison of the numbers of rickettsiae-free macrophages immediately and 24 hr after infection, for each time of lymphokine treatment, strongly suggested that the major mechanism for suppression of rickettsial infection in lymphokine-pretreated macrophages was a decrease in the ability of rickettsiae to enter macrophages. However, the percentage of rickettsiae-free macrophages in lymphokine-treated cultures 24 hr after infection was slightly but consistently greater than that observed immediately after infection, which suggested that intracellular killing of rickettsiae by lymphokine activated macrophages may occur also. This possibility was examined by analysis of rickettsial infection of macrophages treated with lymphokines after infection.

Rickettsial infection of macrophage cultures treated with lymphokines after infection. Macrophage cultures were exposed to viable rickettsiae for 1 hr, washed, and then incubated in dilutions of lymphokines for 24 hr. Rickettsiae-free macrophages were significantly increased in cultures treated with lymphokines after infection. Microbicidal activity was evident through a 1:40 dilution of active supernatants. The time course for induction of macrophage rickettsiacidal activity was examined by exposing macrophage cultures to viable rickettsiae for 1 hr, washing, and then incubating in a 1:10 dilution of lymphokines for various times through 24 hr. Cultures were examined microscopically for percentage of infected macrophages 24 hr after exposure to rickettsiae. Rickettsiacidal activity was detected as early as 1 hr after lymphokine treatment with maximal levels being evidenced within 4 hr of lymphokine treatment.

Treatment of macrophage cultures with lymphokines 4 hr prior to the exposure to viable rickettsiae reduced the number of viable intracellular rickettsiae recovered in the macrophage lysate to 40% of that recovered from medium-treated cells (Table 17). The increase in intracellular killing of lymphokine-activated macrophages was also confirmed by plaque titration of rickettsiae. The number of viable rickettsiae recovered from infected macrophages treated with lymphokines for 18 hr after infection was reduced to 80% of that recovered from medium-treated cells. The largest drop in titer of intracellular rickettsiae occurred in macrophage cultures activated by lymphokines prior to infection and reincubated in lymphokines for 18 hr. These cultures demonstrated a 90% decrease in viable rickettsiae.

Table 17. Titration of viable rickettsiae in lysed peritoneal cell cultures by plaque assay in irradiated L929 cell monolayers.

Sample Time	Peritoneal Cell Culture Treated With	Plaque Titer ($\times 10^4$)	Reduction Viable Rickettsiae:
EXPERIMENT 1:			
Immediate	Medium	16.0 ± 1.5	
	Lymphokines prior to infection	6.0 ± 0.3	38%
18 hour	Medium	14.6 ± 1.2	
	Lymphokines prior to and after infection	1.2 ± 0.2	92%
EXPERIMENT 2:			
Immediate	Medium	7.0 ± 0.3	
	Lymphokines prior to infection	4.3 ± 0.2	37%
18 hour	Medium	5.9 ± 0.4	
	Lymphokines after infection	1.3 ± 0.0	78%
	Lymphokines prior to and after infection	0.7 ± 0.1	88%
EXPERIMENT 3:			
Immediate	Medium	10.4 ± 1.2	
	Lymphokines prior to infection	6.0 ± 0.3	39%
18 hour	Medium	9.2 ± 0.2	
	Lymphokines after infection	2.2 ± 0.6	76%
	Lymphokines prior to and after infection	0.9 ± 0.0	91%

The data clearly demonstrated that soluble factors in culture supernatants of antigen-stimulated spleen cells of rickettsial-infected mice activate resident peritoneal macrophage populations to kill R tsutsugamushi, strain Gilliam. Analysis of results suggested that this rickettsiacidal activity was mediated by at least 2 mechanisms: (1) decreased infectivity of rickettsiae for lymphokine-treated macrophage cultures, and (2) increased intracellular killing of viable rickettsiae by lymphokine-activated cells.

Changes in the macrophage cell membrane occur during activation by immune reactions in vivo or by lymphokine treatment in vitro. These changes in the macrophage cell membrane include alteration in the glycocalyx composition (36), cell surface antigens (37), enzyme activities and Fc and complement receptors (38). Alteration in numbers or affinity of macrophage receptor sites for rickettsial penetration by membrane changes induced after lymphokine activation could account for the 40% reduction in viable intracellular organisms recovered from lymphokine-treated cells immediately after infection.

Decreases in rickettsial infectivity also could result from elaboration of antibacterial factors by lymphokine-activated macrophages which kill or metabolically compromise rickettsiae in the extracellular environment. Such antibacterial factors are responsible for the killing of Listeria monocytogenes (39,40,41) and Mycobacterium bovis (42) in cultures of macrophages activated in vivo as a consequence of chronic infection, and by antigen of mitogen-induced immune responses. The techniques used in this report cannot readily distinguish between these alternative mechanisms for decreased infectivity; therefore, additional studies are needed to define this observation.

Increased intracellular killing of viable rickettsiae by lymphokine-activated macrophages was evidenced by the number of viable rickettsiae recovered from infected macrophages activated by lymphokines after infection being reduced to 70 to 80% of that recovered from control macrophages. Increased microbicidal activity of activated macrophages has been documented in other systems and includes a broad range of pathogens from viruses to protozoa (27,28,29,43,44,45). Interactions previously described for activated macrophages with most of the facultative or obligate intracellular parasites, including Coxiella burnetii, were dependent upon changes in the rate of phagocytosis and phagolysosomal degradation. This was not the case with other rickettsiae, including R tsutsugamushi, which escape normal phagolysosomal degradation processes. After penetration, these organisms multiply freely in the cytoplasm of infected leukocytes (46).

Rickettsiacidal activity of activated macrophages, therefore, must represent effects of intracytoplasmic killing mechanisms distinct from the potent enzyme systems of phagolysosomal digestion. The nature of this microbicidal mechanism within the cytoplasmic matrix currently is unknown. A recent report by Nogueira and Cohn (44) suggested that cytoplasmic killing of intracellular parasites by activated macrophages may not be limited to rickettsiae. Macrophages treated with factors from antigen-stimulated spleen cells displayed strong microbicidal activity against trypomastigotes of Trypanosoma cruzi. Increased microbicidal activity of macrophages treated with these factors prior to infection was secondary to changes induced within the phagolysosomal system. If, however, trypomastigotes were allowed to escape into the cytoplasm of the cell and spleen cell factors were added only after infection, macrophages were also able to kill the trypomastigotes.

It should be emphasized that despite our documentation of two mechanisms by which activated macrophages maintain themselves in an uninfected state, it was not possible to completely protect macrophage cultures from rickettsial infection by treatment with lymphokine-rich supernatants. Lymphokine treatment prior to and after infection reduced numbers of viable rickettsiae in lysed peritoneal cell cultures to 90% of that recovered from control cultures. These residual rickettsiae were consistent with the heterogeneity of responses throughout rickettsiae-macrophage interactions. Only 20 to 40% of the resident macrophage population initially exhibited intracellular rickettsiae following exposure to a range of viable organisms. With a 10-fold increase in multiplicity of infection (3 to 30 PFU rickettsiae/macrophage) an increase in infected macrophages was not observed (Table 15). This range of infection (20 to 40%) in normal macrophages probably reflects the proportion of susceptible cells present in the peritoneum at the time of macrophage harvest. Heterogeneity also was evident in macrophage responsiveness to lymphokine activation (47). Such heterogeneous responses may provide a cellular basis for the clinical observation that viable rickettsiae may be isolated from lymph node biopsies for up to 1 yr following recovery from scrub typhus (48).

6. Host defenses in experimental scrub typhus: mapping of the gene that controls natural resistance in mice.

Inbred mouse strains differ dramatically in their natural resistance to a number of infectious agents. Susceptibility to certain leukemogenic viruses has been linked to the major histocompatibility complex (H-2)³ and may reflect expression of the immune response (49). However, resistance to a variety of bacterial (50,51,52), viral (53)

and protozoal (54,55) infections has been found to be under the control of genes outside the H-2 complex and represent resistance mechanisms other than those involving specific immune recognition.

The causative agent of human scrub typhus, Rickettsia tsutsugamushi, is an obligate intracellular parasite that also causes lethal infections in certain mouse strains. We recently reported that natural resistance in mice to the Gilliam strain of the organism was controlled by a single, autosomal, dominant gene not linked to the H-2 complex (56).

In the present investigation, we have continued these studies and attempted to map the chromosomal location of this gene. Using recombinant inbred (RI) strains of mice, we have found that this gene is located on chromosome 5, closely linked to the retinal degeneration (rd) locus and have confirmed this with a backcross linkage analysis. We have assigned to it the designation Ric, with r and s representing the resistance and susceptible alleles respectively.

Mice. Inbred CeH/HeJ, DBA/2J, C57BL/6J and C57BL/6J-le (light ears) mice were obtained from the Jackson Laboratory, Bar Harbor, ME and were between 6 and 12 weeks of age when used in experiments. Recombinant inbred mouse lines were produced at the Jackson Laboratory by inbreeding randomly chosen pairs of mice from the F₂ generation of the cross between 2 dissimilar progenitor strains (57). The BXD RI strains were derived from the initial cross of C57BL/6J with DBA/2J (58), BXH RI strains from the cross of C57BL/6J with C3H/HeJ (59), and the BXJ RI strains from the cross of C57BL/6J with SJL/J (60). The progenitor strains of the 3 RI lines carried differing Ric alleles (56). All RI lines had attained at least the F₂₀ generation of inbreeding when tested for genetic markers. Backcross progeny mice were obtained from (C57BL/6J x DBA/2J)F₁ males backcrossed to DBA/2J females and from (C57BL/6J x C3H/HeJ)F₁ males backcrossed to C3H/HeJ females.

Rickettsia tsutsugamushi resistance. Mice were tested for the presence of the resistant allele, Ric^r by inoculating intraperitoneally (ip) 1000 50% mouse infectious doses (MID₅₀) of the Gilliam strain of R tsutsugamushi. Mice surviving 28 days after original challenge were rechallenged with 1000 MLD₅₀ of the Karp strain of R tsutsugamushi to establish the presence of immunity as an assurance that survivors had received the original Gilliam dose. All mouse strains used in this study are susceptible to Karp infection and are killed by ip challenge of 10 MLD₅₀ (56).

Mice surviving both challenges were considered to carry the Ric^r allele. Mice that died from the initial challenge were considered to be homozygous Ric^s/Ric^s, since previous studies established the dominance of the Ric^r allele (56). The Gilliam (166th egg passage) and Karp (51st egg passage) strains were propagated, stored, and quantified by methods previously reported (11). The MLD₅₀ determinations were calculated using C3H/HeJ mice by methods previously described (56).

Retinal degeneration determination. Retinal degeneration is a recessive gene which can be assessed using histological criteria (61). Mice (4-8 weeks old) were anesthetized and 1 eye was removed, fixed in 10% buffered formalin solution, sectioned, and stained with hematoxylin and eosin. Mice lacking 2 or more of the outer most divisions of the inner transparent layer of the retina were classified as homozygous rd/rd.

Enzyme testing. The isoenzymes, a and b, coded by the phosphoglucomutase-1 (Pgm-1) locus on chromosome 5 were determined by starch gel electrophoresis of heparinized blood. Mouse liver homogenates were typed for the quantitative variants of glucuronidase (Gus) b and h, using a colorimetric procedure.

Estimation of recombination frequencies. Recombination frequencies (r) are indicators of the relative distance between 2 linked genes on a chromosome and were estimated for backcross data by $r = o/n$, where o is the observed number of recombinants and n is the number of backcrosses contributing to the estimate. For RI strain data, recombination frequencies were estimated using an equation derived by Halden and Waddington (62), which relates the probability of fixing a recombinant genotype in an RI strain (R) to the recombination frequency (r); $R = 4r/(1 + 6r)$. r is estimated by $r/(4 - 6R)$, where R is estimated from RI data. The variance of the estimated r is $r(1 + 2r)(1 + 6r)^2/4n$, where n is the number of RI strains contributing to the estimate.

Response of inbred mouse strains to R tsutsugamushi. Three strains C57BL/6J, C3H/HeJ, and DBA/2J were chosen for analysis because they were either resistant or susceptible (56) and the progenitors of 2 large sets of RI mouse strains (58,59). C57BL/6J mice were resistant to at least 10⁵ MLD₅₀; whereas, all of the C3H/HeJ and DBA/2J mice were killed by 10² MLD₅₀. A dose of 10³ MLD₅₀ was, therefore, selected to assess the susceptibility of mouse strains in the study.

Response of RI mouse strains to R tsutsugamushi. Two sets of RI lines were used to map Ric. Three mice per strain were used, and only strains showing 100% survival or resistance in an individual trait were included. Twenty-four different BXD strains were infected ip with 10^3 MLD50 of R tsutsugamushi; 12 strains were resistant and 12 were susceptible to infection (Table 18). An analysis of the previously mapped chromosomal markers in the BXD RI lines suggested resistance was possibly linked to chromosome 5, locus Pgm-1. In only 9 of the 24 BXD strains did the appropriate Ric and Pgm-1 alleles of the progenitor parent strains fail to segregate together. If there had been no linkage, a crossover rate of approximately 50% (12 of 24) would have been expected.

To confirm this linkage, the resistance pattern was analyzed of 13 BXH strains, which had been typed for Pgm-1 and 2 additional chromosome 5 markers, Gus and rd. Four strains were resistant and 9 susceptible to R tsutsugamushi (Table 19). The pattern of resistance in these strains confirmed the linkage to Pgm-1. Further, the data suggested that Ric was closely linked to rd, since there were no crossovers between Pgm-1 and Ric and 5 crossovers between Gus and Ric.

The BXJ-1 and BXJ-2 strains from a third RI line were tested for susceptibility because progenitor strains (C57BL/6J and SJL/J) were known to differ in rickettsial susceptibility, as well as the chromosome markers of rd and Pgm-1. No crossovers between Ric and either loci were observed.

Backcross linkage analysis. A backcross linkage analysis was performed to obtain a better estimate of the distance between Ric and selected chromosome 5 markers and to possibly establish the correct gene order for these 2 loci. (C57BL/6J x DBA/2J) F_1 males were backcrossed to DBA/2J females and the progeny were typed for Pgm-1, then tested for resistance to R tsutsugamushi. Of the 56 mice tested, 27 resistant mice possessed the Pgm-1 genotype of the resistant F_1 parent (a/b), while 15 susceptible mice possessed the genotype of the susceptible DBA/2J parent (b/b) (Table 20). Those mice that possessed the resistant Pgm-1 genotype and succumbed to rickettsial infection or possessed the homozygous susceptible (b/b) genotype and resisted lethal infection were considered to be recombinants. Only 14 recombinant events were observed. These findings confirmed the linkage of Ric to Pgm-1.

To estimate the distance between Ric and rd, (C57BL/6J x C3H/HeJ) F_1 males were backcrossed to C3H/HeJ females and the progeny were tested for these 2 loci. Of the 93 progeny tested, 46 resistant mice and 44 susceptible mice retained the rd genotype of the

Table 18. Susceptibility of BXD recombinant inbred mice to R tsutsugamushi, strain Gilliam.

Locus ^a	C57BL/6J	DBA/2J	BXD Strain																									
			(Resistant)																									
			1	6	8	9	13	15	19	20	23	25	27	28														
<u>Pgm-1</u>	B	D	B	B	B	D	B	B	D	D	D	B	B	D														
						X			X	X	X																	
<u>Ric</u>	B	D	B	B	B	B	B	B	B	B	B	B	B	B														
			2	5	11	12	14	16	18	21	22	24	29	30														
<u>Pgm-1</u>	B	D	D	D	B	B	B	D	B	D	D	D	D	D														
					X	X	X		X																			
<u>Ric</u>	B	D	D	D	D	D	D	D	D	D	D	D	D	D														

^a "B" and "D" are used as generic symbols for alleles inherited from the C57BL/6J and DBA/2J strains, respectively. The C57BL/6J genotype is Pgm-1a Ric^r/Pgm-1a Ric^r, and this strain is resistant. The DBA/2J genotype is Pgm-1b Ric^s/Pgm-1b Ric^s, and this strain is susceptible. The letter "X" indicates the occurrence of a crossover between the adjacent loci.

Table 19. Susceptibility of BXH recombinant inbred mice to R tsutsugamushi, strain Gilliam.

Locus ^a	C57BL/6J	C3H/HeJ	BXH Strain														
				Resistant								Susceptible					
				6	10	11	12	2	3	4	5	7	8	9	14	19	
<u>Pgm-1</u>	B	H		B	H	H	B	H	H	H	B	B	H	H	H	H	
<u>Ric</u>	B	H		X	X	X					X	X					
<u>rd</u>	B	H		B	B	B	B	H	H	H	H	H	H	H	H	H	
<u>Gus</u>	B	H		B	B	B	B	H	H	H	H	H	H	H	H	H	
				X	X	X		X			X	X			X		
				B	H	B	B	B	H	H	B	B	H	H	B	H	

^a "B" and "H" are used as generic symbols for alleles inherited from the C57BL/6J and C3H/HeJ strains, respectively. The C57BL/6J genotype is Pgm-1a Ric^r + Gusb/Pgm-1a Ric^r Gusb. The C3H/HeJ genotype is Pgm-1b Rics rd Gush/Pgm-1b Rics rd Gush. The letter "X" indicates the occurrence of a crossover between adjacent loci.

Table 20. Summary of backcross linkage analysis between Ric and Pgm-1^a.

<u>Pgm-1</u> Genotype	No. of Resistant Mice (<u>Ric^r</u> / <u>Ric^s</u>)	No. of Susceptible Mice (<u>Ric^s</u> / <u>Ric^s</u>)
<u>b/b</u>	5	15
<u>a/b</u>	27	9

^a (C57BL/6J x DBA/2J)F₁ mice were backcrossed to DBA/2J, and the progeny were typed for Pgm-1 and Ric. C57BL/6J is Pgm-1^a and Ric^r, and DBA/2J is Pgm-1^b and Ric^s. Backcross mice were either homozygous Pgm-1^b/Pgm-1^b or heterozygous Pgm-1^a/Pgm-1^b at this locus.

Table 21. Summary of backcross linkage analysis between Ric and rd^a.

<u>rd</u> Genotype	No. of Resistant Mice (<u>Ric^r</u> / <u>Ric^s</u>)	No. of Susceptible Mice (<u>Ric^s</u> / <u>Ric^s</u>)
<u>rd/rd</u>	0	44
<u>+/rd</u>	46	3

^a (C57BL/6J x C3H/HeJ)F₁ mice were backcrossed to C3H/HeJ females, and the progeny were tested for rd and Ric. The genotype for rd and Ric markers, respectively, are: C57BL/6J, +r/+r, C3H/HeJ, rd s/rd s. Backcross mice were either homozygous rd/rd or heterozygous rd/+ at this loci.

respective resistant F₁ parent (rd/+) or susceptible C3H/HeJ parent (rd/rd) (Table 21). Three susceptible mice possessed the resistant parental genotype (+/rd) and were the only observed crossovers.

Two, three-point cross analyses were performed to determine the gene order. Twenty-four of the backcross progeny listed in Table 21 also were tested for Gus in addition to rd and Ric. Seven crossovers between Gus and the rd and Ric loci were found (Table 22). However, gene order could not be determined since crossovers between rd and Ric were not observed. In the second three-point analysis, 34 of the backcross progeny listed in Table 21 were tested for Pgm-1, as well as Ric and rd. Six crossovers occurred between Pgm-1 and rd and 8 crossovers between Pgm-1 and Ric (Table 23). More importantly, 2 crossovers between rd and Ric were observed. In 2 susceptible mice, the resistant Pgm-1 genotype remained linked to the resistant rd genotype suggesting the gene order Pgm-1-rd-Ric-Gus since reversal of rd and Ric would postulate double crossovers.

Response of the C57BL/6J-LE to R tsutsugamushi. The C57BL/6J-le mouse is a congenic strain which carries the C3H/HeJ chromosome 5 alleles for rd, le, and Gus on a C57BL/6J background. The Pgm-1 locus is of the C57BL/6J type. Three C57BL/6J-le mice were tested for R tsutsugamushi susceptibility in 2 separate experiments and were found to be resistant. These data imply a gene order of Pgm-1-Ric-rd-le-Gus, since transposing Ric and rd would postulate a double crossover between the closely approximated rd and le loci. This gene order is in marked contrast with that suggested by the results shown in Table 23 above.

The data presented clearly demonstrated that Ric, the gene controlling resistance to lethal scrub typhus infections in mice, was located on chromosome 5 and was closely linked to the rd locus. The recombination frequency (r) between rd and Ric was 0.0323 (3/93) in the backcross progeny. No recombinants between Ric and rd were found among 15 RI strains. Thus, we calculated r to be \bar{r} 0.0172, using the Haldane and Waddington formula (62) and assuming that the next RI strain observed would have been a crossover (1/16).

Experiments to define gene order yielded conflicting results. We believe the most reliable data were derived from the congenic C57BL/6J-le mouse strain and the the gene order is probably Pgm-1-Ric-rd-Gus. In the three-point crosses employing the Pgm-1, rd, and Ric markers, the only recombinants were 2 susceptible animals which carried the resistant parent alleles at the other loci,

Table 22. Segregation of Gus, rd, and Ric markers in backcross
(C57BL/6J x C3H/HeJ)F₁ x C3H/HeJ^a.

Region of Recombination	Genetic Locus			No. of Mice
	<u>rd</u>	<u>Ric</u>	<u>Gus</u>	
None	+	<u>r</u>	<u>b</u>	18
	<u>rd</u>	<u>s</u>	<u>h</u>	19
<u>rd-Ric</u>	+ X	<u>s</u>	<u>h</u>	0
	<u>rd</u> X	<u>r</u>	<u>b</u>	0
<u>Ric-Gus</u>	+	<u>r</u> X	<u>h</u>	4
	<u>rd</u>	<u>s</u> X	<u>b</u>	3
<u>rd-Ric-Gus</u>	+ X	<u>r</u> X	<u>b</u>	0
	<u>rd</u> X	<u>r</u> X	<u>h</u>	0
Total				44

^a The parental genotypes for rd, Ric, and Gus markers, respectively, are C3H/HeJ, rd s h/rd s h; C57BL/6J, + r b/+ r b; F₁, rd s h/+ r b.

Table 23. Segregation of Pgm-1, rd, and Ric markers in backcross (C57BL/6J x C3H/HeJ) F_1 x C3H/HeJ^a.

Region of Recombination	Genetic Locus			No. of Mice
	<u>Pgm-1</u>	<u>rd</u>	<u>Ric</u>	
None	<u>a</u>	+	<u>r</u>	12
	<u>b</u>	<u>rd</u>	<u>s</u>	14
<u>Pgm-1 - rd</u>	<u>a</u> X	<u>rd</u>	<u>s</u>	3
	<u>b</u> X	+	<u>r</u>	3
<u>rd-Ric</u>	<u>a</u>	+ X	<u>s</u>	2
	<u>b</u>	<u>rd</u> X	<u>r</u>	0
<u>Pgm-1-rd-Ric</u>	<u>a</u> X	<u>rd</u> X	<u>r</u>	0
	<u>b</u> X	+	<u>s</u>	0
Total				34

^a The parental genotypes for the Pgm-1, rd, and Ric markers, respectively, are: C3H/HeJ, b rd s/b rd s; C57BL/6J, a + r/a + r; F_1 , a + r/b rd s

suggesting a gene order of Pgm-1-rd-Ric. The possibility that these latter apparent recombinants were genetically resistant mice which died from other causes or from R tsutsugamushi infection following a compromise of the innate resistance by undefined mechanisms cannot be excluded. More compelling evidence for the latter gene order would have been a resistant backcross mouse exhibiting susceptible parent alleles for Pgm-1 and rd; however, this was never observed.

The gene product of the Ric locus and the mechanism of its action remains unknown. The Ric locus does not control the ability of R tsutsugamushi to replicate within cultured cells because these organisms have been propagated equally well in fibroblasts derived from resistant or susceptible mice (56). Further, an alteration of the immune responsiveness of susceptible mice apparently does not occur as susceptible mice can be protected against subsequent ip infection by a subcutaneous inoculation with virulent R tsutsugamushi organisms (56). This finding also demonstrates that susceptible mice possess the appropriate cellular components (i.e., macrophages, granulocytes, and immunoglobulin) to control rickettsial infection. The only difference between susceptible and resistant strains reported to date is that peritoneal macrophages of susceptible mice are killed during in vivo infection while those of resistant mice survive (63). Extrapolation from studies of other investigators (64,65) suggests that the Ric locus may control the ability of mice to develop acute and/or chronic inflammatory responses, which would affect directly the ability of an animal to resist rickettsial infection. The chronic granulomatous reaction of mice to killed BCG organisms was shown to be controlled by a non-H-2 linked gene, and the mouse strain distribution of responsiveness to this BCG effect correlated with that of Ric (64). Similarly, a correlation was reported in the ability of mice to develop an elevated serum complement level in response to an acute inflammatory reaction (65).

There does not appear to be a correlation between Ric and the genes that control natural resistance to several other infectious organisms. The gene that controls resistance of mice to Leishmania donovani (Lsh) was mapped to chromosome 1 (54), and the resistance patterns among inbred mouse strains to Salmonella typhimurium (52), Listeria monocytogenes (50), Corynebacterium kutscheri (51), Toxoplasma gondii (55), and Herpes simplex virus (53) do not correspond to that observed for Ric (56). Furthermore, the Ric locus does not control resistance to all R tsutsugamushi strains, since all strains of mice have been found to be equally susceptible to the Karp strain of R tsutsugamushi (56). It would appear, therefore, that the mechanism of resistance for Gilliam strain infection differs significantly at the genetic

level from the mechanism of resistance to other microorganisms, to include other strains of R tsutsugamushi.

The only gene that maps close to Ric that may be involved in resistance is a minor histocompatibility locus H(go). Williams et al. (55) found that one of the genes controlling resistance to T gondii was linked to a minor histocompatibility locus, H-13; therefore, it is possible that resistance genes and minor histocompatibility loci form gene complexes that involve the host defense mechanism in a manner similar to the relationship between the mouse H-2 complex and the immune response. In any event, there exists in the mouse a number of non-H-2 linked resistance genes of which Ric is but one. Identification of these genes and their products will be invaluable in furthering an understanding of host resistance to infectious organisms.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
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A. PRIMARY	62770A	3M762770A802		00		007	
B. CONTRIBUTING							
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11. TITLE (Precede with Security Classification Code) ^a							
(U) Field Studies of Rickettsioses and Other Tropical Diseases							
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17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
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20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME * Walter Reed Army Institute of Research				NAME * US Army Medical Research Unit Malaysia			
ADDRESS * Washington DC 20012				ADDRESS * Kuala Lumpur, Malaysia			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Pursuit SSAN if U.S. Academic Institution)			
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22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Shirai, Akira, PhD			
				NAME: Dohany, Alexander L., MAJ, MSC			
23. KEYWORDS (Precede EACH with Security Classification Code) (U) Scrub typhus; (U) R. tsutsugamushi; (U) Malaysia; (U) Epidemiology; (U) Ecology; (U) Serology; (U) Leptotrombidium spp; (U) Pathogenesis							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Pursuit individual paragraphs identified by number Precede text of each with Security Classification Code)							
23(U) 1. Investigate the epidemiology of scrub typhus in human populations; 2. Study the chigger vector of scrub typhus; 3. Determine the strains of R. tsutsugamushi causing human infections; 4. Evaluate new treatments for scrub typhus; 5. Study the basis of scrub typhus immunity; 6. Develop a laboratory animal model for scrub typhus. These studies are designed to eliminate scrub typhus as a military medical problem.							
24(U) 1. Evaluation of clinical, pathological, and serological characteristics of scrub typhus in rural populations. 2. Determine changes in chigger populations due to changes in ecology. Identify R. tsutsugamushi infection in vector chiggers. 3. Antigenically characterize R. tsutsugamushi strains isolated throughout the endemic region.							
4. Administer single dose doxycycline in early febrile disease. 5. Test antigen-stimulated, immune lymphocytes for blastogenesis. 6. Characterize R. tsutsugamushi infection in cynomolgus monkeys.							
25(U) 78 10 - 79 09 1. Large rural populations having a high incidence of scrub typhus have been identified which can be used to study chemotherapy, chemoprophylaxis, and vaccine prophylaxis. 2. Vector chigger populations decline as oil palm areas mature. Eight chigger species were found to be infected for the first time. 3. R. tsutsugamushi strains within the endemic area were found to be predominately Karp-related.							
4. Early doxycycline treated patients did not suffer apparent relapses. 5. Methods for transporting lymphocytes from field study sites while maintaining their viability and reactivity have been perfected. 6. Studies have shown the cynomolgus monkey to be an excellent model for scrub typhus. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.							

^aAvailable to contractors upon originator's approval

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DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE DD FORMS 1498A 1 NOV 66 AND 1498-1 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

Project 3M762770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 007 Field Studies of Rickettsioses and Other Tropical Diseases

Investigators:

Principals: LTC David L. Huxsoll, VC; LTC Michael G. Groves, VC
Associate: Dr. Akira Shirai, Ph.D.; LTC J. P. Saunders, RAMC;
MAJ Alexander L. Dohany, MSC; CPT Robert M. Werner, VC; CPT Gregory B. Heisey, VC; Miss Elsie Gan, B.S.

THE IMPACT OF ECOLOGICAL CHANGES ON THE POPULATION OF SCRUB TYPHUS VECTORS IN AN AREA CLEARED OF FOREST AND REPLANTED IN A TYPE OF VEGETATION THAT NATURALLY SUPPORTS CHIGGER POPULATIONS

Scrub typhus has long been associated with specific types of habitat. The name scrub typhus was coined to associate the disease with the scrub terrain where the disease was often contracted. Within Peninsular Malaysia, the vector mites which transmit the disease can be found in lalang fields, along sandy beaches, in different forest types, and within major agricultural crops.

Since early 1960, hundreds of thousands of acres of land in Malaysia have been put into agricultural production primarily through large government-financed programs. The Federal Land Development Authority (FELDA), established in 1956, is now one of the largest land development agencies of the Malaysian Government. FELDA operations include land clearing, planting of main crops (primarily oil palm and rubber), development of villages, selection and emplacement of settlers, management of projects, provision of credit, marketing services, and facilitating social and community development.

During the development stage FELDA contractors carry out jungle clearing, plant the main crop, and maintain it until settlers enter the project. Settler houses are also built by FELDA contractors. Roads, water supplies, schools, and clinics are constructed by the Public Works Department. Settlers enter the project 2½-3 years after the start of land clearing and are immediately responsible for weeding, fertilizing, and the control of plant pests and diseases. Prior to the time that the settler receives income from his land, FELDA provides subsistence credits to the settler. Approximately 5 years after planting of oil palm (later in the case of rubber), the settler commences repaying the government loan which covers the cost of land development,

fertilizers, subsistence credits, and housing. The settler acquires title to the land (or a share in a cooperative) after about 15 years of loan payment.

FELDA oil palm development schemes each consist of approximately 4000 acres. Approximately 400 settler families enter each scheme with 10 acres of oil palm allotted to each settler family.

The oil palm trees are planted in rows approximately 6 meters apart. Before the trees begin to bear fruit, grasses are allowed to grow around the trees. In order to harvest the fruit, a frond below the fruit must be cut. These fronds are stacked between every other row of trees forming litter piles (Figure 1). Thus, rows are separated alternately by grass strips and litter piles. As the settlers begin working their plot, some of the grasses are eliminated due to cultivation practices. Also, as the trees grow a canopy forms reducing the sunlight which reaches the ground around the trees. This, in turn, causes many of the grasses to be eliminated.

Previous studies at a district hospital and a rural health center provided data which showed that scrub typhus was a common cause of fever among oil palm workers (1). Studies at the Bukit Mendi FELDA oil palm development scheme, which contained plantings of 5 and 7 years of age, provided data which served to suggest a greater risk of infection in the younger oil palm (WRAIR Annual Progress Report, 1 Oct 77 - 30 Sep 78). The major difference in the 2 groups of oil palm was the presence of dense grasses between the rows of young oil palm trees and the absence of such grasses in the older oil palm. A natural succession of plants and micro-habitats seemed to exist in the oil palm.

Because these changes within the micro-habitats might have an effect upon the distribution of the chiggers in the areas and, in turn, upon the chances of human contact with the vectors, we undertook a longitudinal study of a number of schemes of varying ages within the Jengka Triangle complex.

Results: At the beginning of the study in April, 1977, the Jengka Triangle complex (Figure 2) consisted of 23 schemes, in which approximately 63% of the total usable acreage was planted in oil palm and the remainder in rubber. The oil palm development schemes varied in age from newly cleared land to oil palm approximately 10-years old. Eight areas were selected for study. These included: uncut forest, unplanted oil palm or scrub (Jengka 23), 1.5-year-old oil palm (Jengka 23), 2-year-old oil palm (Jengka 17), 2.5-year-old oil palm (Jengka 19), 3.5-year-old oil palm (Jengka 15), 5.5-year-old oil palm (Jengka 11), and 9-year-old oil palm (Jengka 2).

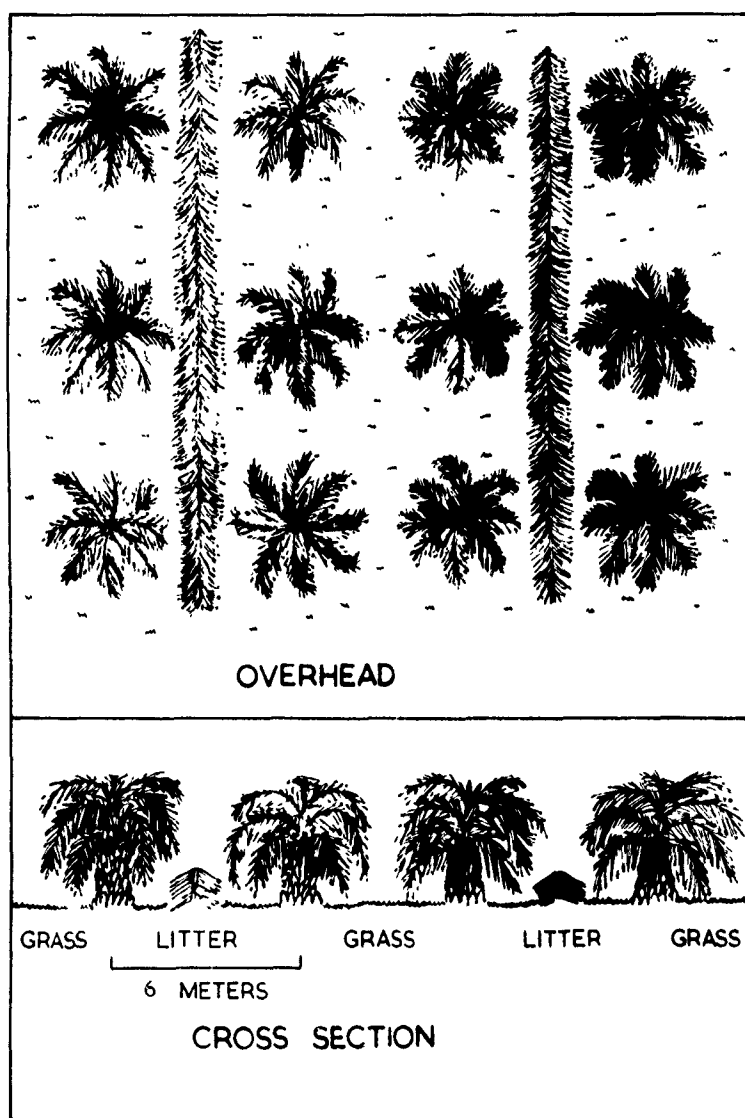


Figure 1. Schematic diagram showing the system of maintaining trees and litter piles within an oil palm scheme.

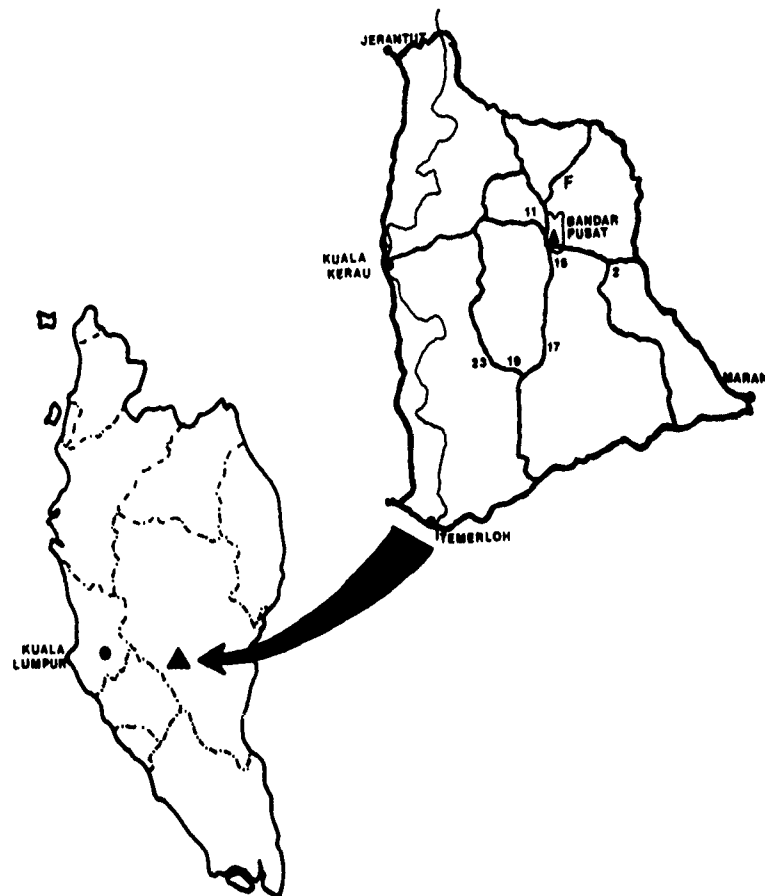


Figure 2. Jengka Triangle Complex, Pahang, Peninsular Malaysia, showing locations of the schemes under study.

Two recently settled schemes (Jengka 11 and Jengka 15) were studied intensively. Febrile illnesses in settlers on these 2 schemes were studied in other projects (see: Scrub Typhus Studies on a Rural Development Complex in Peninsular Malaysia: Antibody Prevalence in Oil Palm Settlers and WRAIR Annual Progress Report, 1 Oct 77 - 30 Sep 78). Settlers resided on Jengka 11 for approximately 18 months and on Jengka 15 for 6 months before the beginning of the study. Numerous cases of scrub typhus had been diagnosed in Jengka 11, but no cases had been diagnosed in Jengka 15.

To correlate changes in chigger populations with human and rodent infections, monthly collections of rodents and chiggers were made from Jengka 11 and Jengka 15. Two permanent trapping grids of 100 traps set 6 meters apart were established in each scheme. Within these grids chiggers were collected from trapped rodents and by the use of black plates (8). Additionally, a "roving" grid was located at different sites each month within the 2 schemes to obtain samples from a larger area. Other "roving" grids were used as time permitted to collect unengorged chiggers by the use of black plates.

Within each of the remaining 6 study areas, 2 permanent trapping grids were established. Rodents were trapped from these grids on a quarterly basis. Chiggers were collected from all trapped rodents for identification, and the rodents were identified, marked, and released at their site of collection. Simultaneously, black-plate collections were made in the same grid areas.

This was a continuing study which began March 1977 and was completed March 1979. Some results have been previously reported (WRAIR Annual Progress Report, 1 Oct 77 - 30 Sep 78). Figure 3 presents the collection results for the first year. A peak in the number of chiggers per Rattus tiomanicus was noted in Jengka 19 (2.5-year-old oil palm at the initiation of the study). In Jengka 2 (a 9-year-old oil palm scheme), L. (L.) deliense made up less than 1% of the chigger population. Most of the chiggers collected from rodents in Jengka 2 were Ascotchoengastia (Laurentella) indica, a nest-dwelling chigger. This represented a dramatic change in the speciation of chiggers.

Figure 4 depicts the collection data for the second year of the study. The peak population occurred in Jengka 23 (1.5-year-old oil palm), and the percentage of L. (L.) deliense was greatly reduced in Jengkas 19, 15, and 11. These data indicated that within an oil palm habitat there is a reduced vector capacity as the oil palm matures. Jengka 23 with the current peak in chiggers and vectors is now the age of Jengka 15 at the start of the study, and Jengka 11 is now approaching the age of Jengka 2 at the start of the study. Changes in the habitat and consequently, in the habits of the hosts have apparently led to these changes in

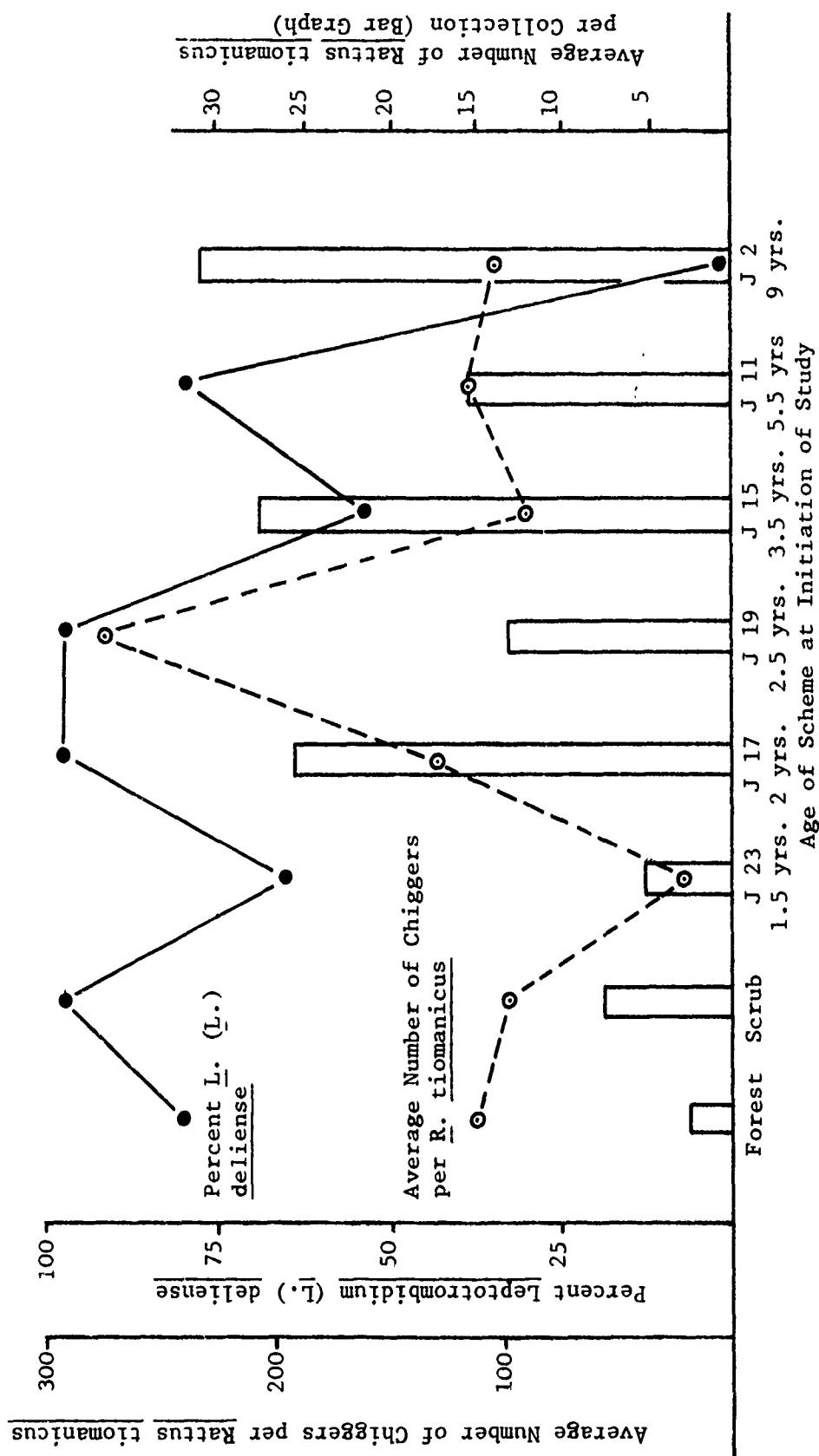


Figure 3. First year density of vector chiggers on the predominant rodent host, *Rattus tiomanicus*, from study sites in Jengka Complex.

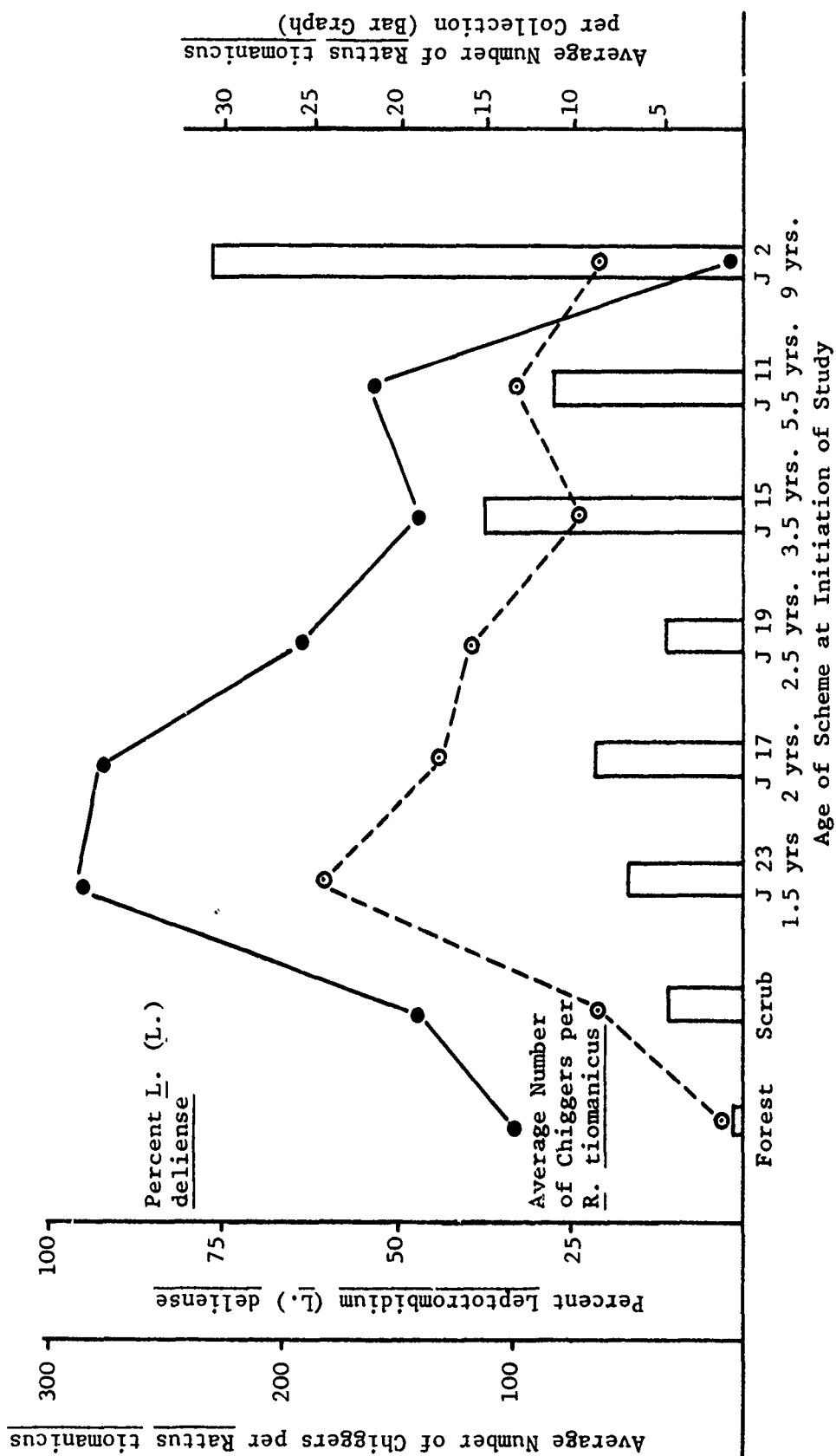


Figure 4. Second year density of vector chiggers on the predominant rodent host, *Rattus tiomanicus*, from study sites in Jengka Complex.

chigger populations. Figures 5 and 6 present the collection of chiggers by month for the 2 intensively studied areas, Jengka 11 and 15. In general, the relative population increases and decreases in L. (L.) deliense paralleled the fluctuations in the total chigger population. Some slight variation in this trend can be noted in the latter part of the study. This decrease in numbers could reflect major changes in the habitats. Environmental and habitat data are currently being analyzed to determine what caused these changes.

Plant composition studies were conducted in collaboration with a plant ecologist from the University of Malaya. These included the identification of the plants within each scheme and the determination of plant density. The number of plant species within the young oil palm schemes (<5.5-years old) was lower than in older schemes; however, the density of grasses was higher. The 2 predominant ground cover grasses Mikania cordata and Paspalum conjugatum found in young oil palm was not common in Jengka 2. Although the number of plant species within Jengka 2 was higher, they provided less ground cover.

During the study, 174 of 4,350 chiggers (4.0%) collected by the black-plate method were found infected with R. tsutsugamushi using the direct fluorescent antibody technique (FAT)(4). Antigenic analyses of these isolates showed the Karp strain to be the overwhelmingly predominant organism, 70.7% (Table 1). No organisms reacting with Gilliam antiserum were identified. Most of the infections within chiggers were monotypic 140/174 (80.5%).

From April 1977 through July 1978, rodents from Jengka 11 and 15 were examined for evidence of R. tsutsugamushi infection. Eight hundred and twenty-three were examined, and R. tsutsugamushi was isolated from 96 (11.7%)(Table 2). Of the positive animals, 60 (15.8%) were from Jengka 11, and 36 (8.1%) were from Jengka 15. For comparison purposes, 200 rodents from Jengka 2 were also examined during August and September 1978 and were found to be negative. This was not surprising, since Jengka 2 also recorded the lowest density of vector chiggers (Figures 3 and 4). Of the 96 rodent isolates, 67 (69.8%) were characterized by the direct FAT (Table 3). The majority of the rodents (94%) had single or multiple infections of Karp or Karp-related strains. Four rodents were infected singly with Gilliam, and 5 had mixed infections of Gilliam and other strains.

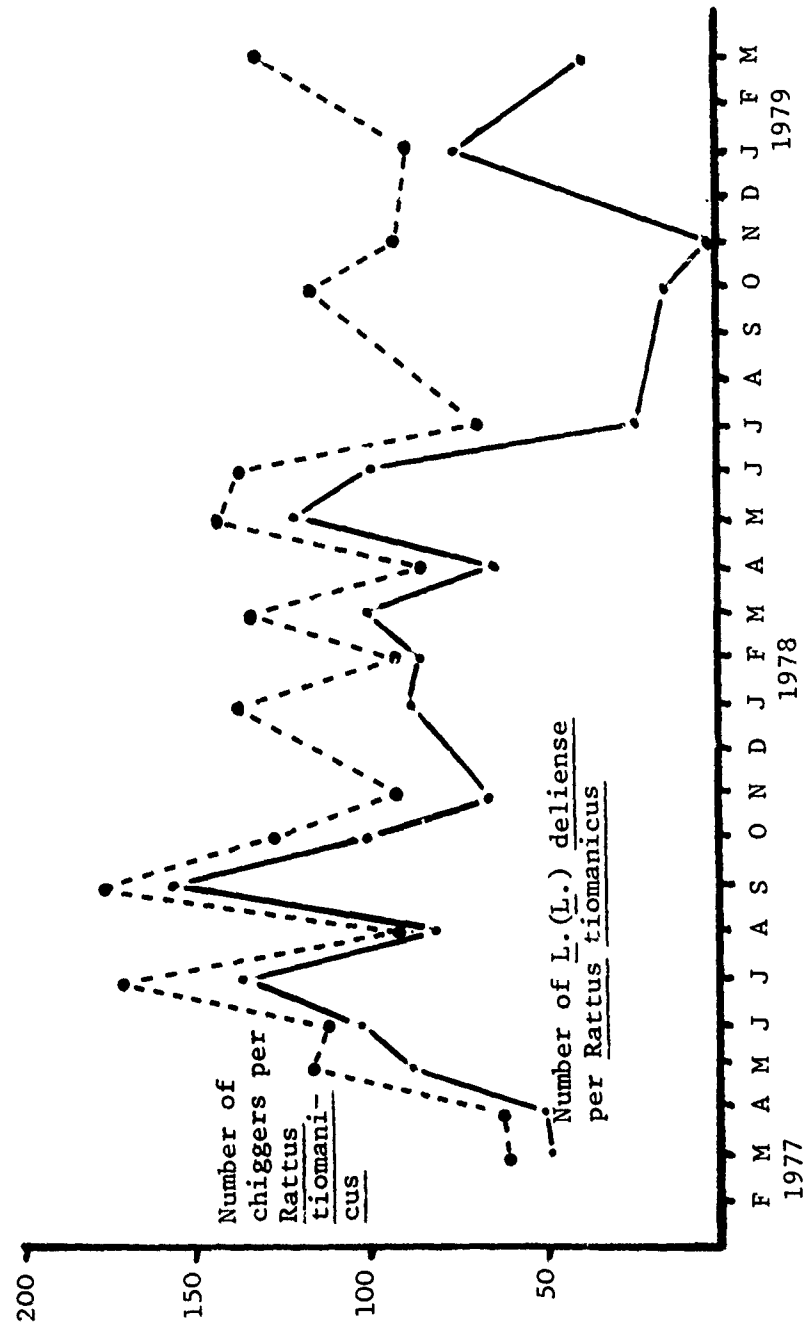


Figure 5. Monthly chigger collections from Jengka 11.

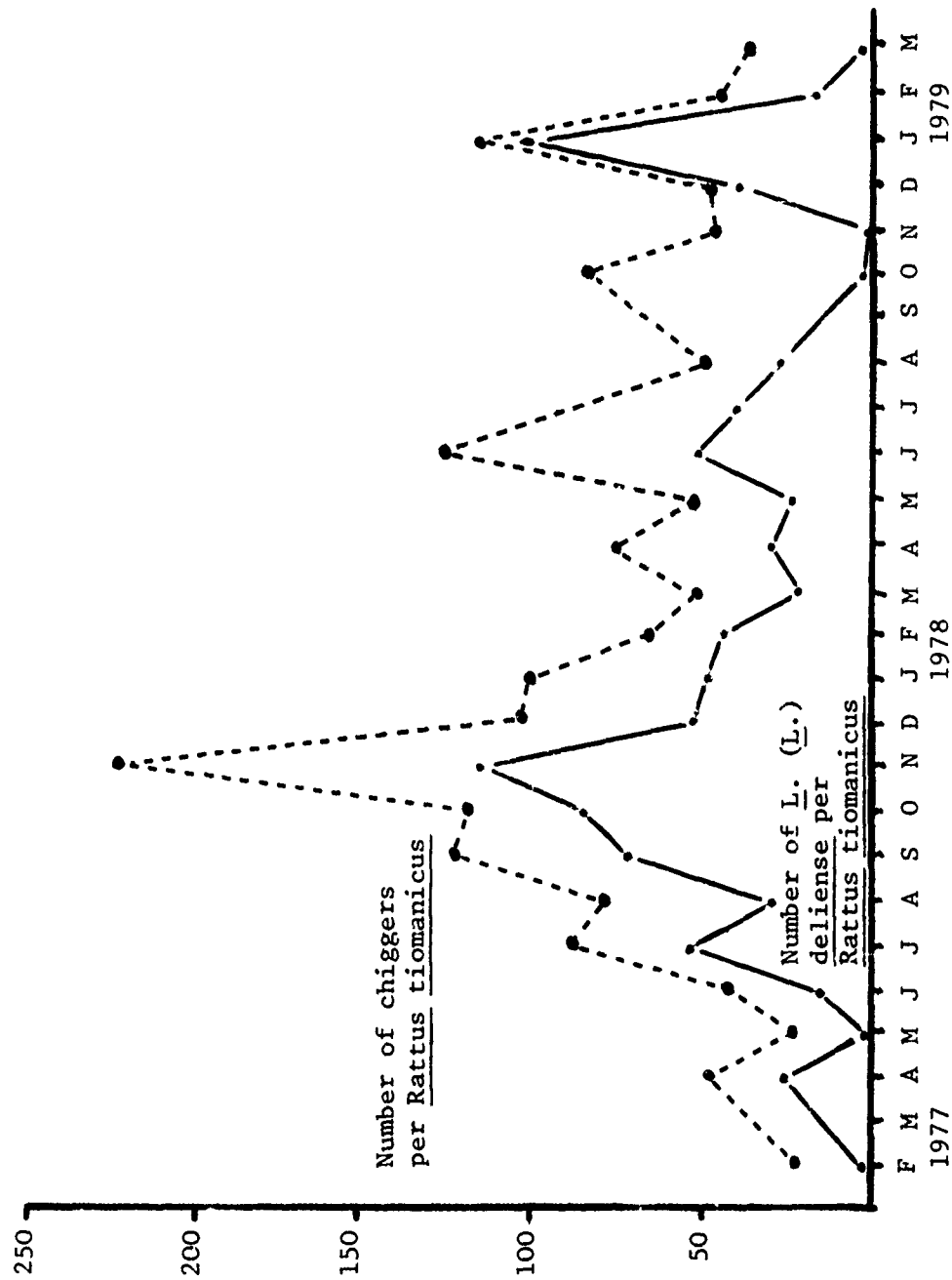


Figure 6. Monthly chigger collections from Jengka 15.

Table 1. Characterization of Rickettsia tsutsugamushi strains found in chiggers collected from Jengka Triangle study sites

Antigenic Combinations	Number	Percent
Karp	123	70.7
TA686	1	.6
TA716	7	4.0
TA763	9	5.2
Karp, TA686	6	3.5
Karp, TA716	9	5.2
Karp, TA763	9	5.2
TA686, TA763	1	.6
TA716, TA763	6	3.5
Karp, TA686, TA716	1	.6
Karp, TA716, TA763	2	1.2
TOTAL	174	100.3

Table 2. Rickettsia tsutsugamushi isolations from rodents in Jengka 11 and Jengka 15.

Date	Jengka 11	Jengka 15	Total
Apr 77	0/15 (0.0)*	-	0/15 (0.0)
May	1/22 (4.6)	1/30 (3.3)	2/52 (3.9)
Jun	1/12 (8.3)	2/58 (3.5)	3/70 (4.3)
Jul	6/30 (20.0)	-	6/30 (20.0)
Aug	4/30 (13.3)	2/30 (6.7)	6/60 (10.0)
Sep	7/30 (23.3)	1/30 (3.3)	8/60 (13.3)
Oct	6/28 (21.4)	2/30 (6.7)	8/58 (13.8)
Nov	3/20 (15.0)	3/30 (10.0)	6/50 (12.0)
Dec	1/10 (10.0)	2/30 (6.7)	3/40 (7.5)
Jan 78	4/30 (13.3)	4/30 (13.3)	8/60 (13.3)
Feb	5/27 (18.5)	5/30 (16.7)	10/57 (17.6)
Mar	9/30 (30.0)	1/30 (3.3)	10/60 (16.7)
Apr	3/9 (33.3)	2/30 (6.7)	5/39 (12.8)
May	4/30 (13.3)	4/30 (13.3)	8/60 (13.3)
Jun	5/30 (16.7)	3/22 (13.6)	8/52 (15.4)
Jul	1/27 (3.7)	4/33 (12.1)	5/60 (8.3)
TOTAL	60/380 (15.8)	36/443 (8.1)	96/823 (11.7)

*Number positive/total observed (% positive).

Table 3. Antigenic characterization of Rickettsia tsutsugamushi isolated from rodents in Jengka 11 and Jengka 15.

Antigenic Combinations	Number of Isolates (% of Isolates)		
	Jengka 11	Jengka 15	Total
Gilliam	3	1	4 (6.0)
TA716	7	3	10 (14.9)
TA763	-	1	1 (1.5)
Karp, TA716	3	3	6 (9.0)
Gilliam, TA716	1	-	1 (1.5)
TA686, TA716	4	4	8 (11.9)
TA716, TA763	2	2	4 (6.0)
TA716, TH1817	-	1	1 (1.5)
Karp, Gilliam, TA716	-	1	1 (1.5)
Karp, TA686, TA716	-	2	2 (3.0)
Karp, TA716, TA763	2	1	3 (4.5)
Karp, TA716, TH1817	-	1	1 (1.5)
Gilliam, TA686, TA716	2	1	3 (4.5)
TA686, TA716, TA763	8	3	11 (16.4)
Karp, Kato, TA686, TA716	1	-	1 (1.5)
Karp, Kato, TA716, TA763	1	-	1 (1.5)
Karp, TA686, TA716, TA763	3	5	8 (11.9)
Karp, Kato, TA686, TA716, TA763	-	1	1 (1.5)
TOTAL	37 (55.2)	30 (44.8)	67 (100.1)

SCRUB TYPHUS STUDIES ON A RURAL DEVELOPMENT COMPLEX
IN PENINSULAR MALAYSIA: ANTIBODY PREVALENCE
IN OIL PALM SETTLERS

Background: Early studies of scrub typhus in Malaya indicated a close association of the disease with land clearance and oil palm plantations (11). One of our earlier studies of a Malaysian oil palm development complex revealed a high incidence of fevers that were attributable to scrub typhus (1). Simultaneous collections of chiggers indicated that vector populations declined as oil palm areas matured (WRAIR Annual Progress Report, 1 Oct 77 - 30 Sep 78). This posed the possibility that human infection rates might vary according to the stage of oil palm growth. Unfortunately, the different phases of oil palm planting in the study area were serviced by the same group of people and verification of this was impossible.

The findings of a recent study on the longevity of antibody to R. tsutsugamushi allow one to accurately estimate attack rates by measuring the point prevalence of antibody (19). This and the location of 3 oil palm areas of close proximity and widely varying ages which are serviced by 3 distinct population groups has allowed us to investigate the relative risk of human infection in oil palm schemes of varying ages. We report the results of these studies here.

Results: Studies were conducted in the FELDA Jengka Triangle area in Peninsular Malaysia. Three settler populations were identified for survey: (1) Jengka 17 (J17) settled for about 1 year, (2) Jengka 11 (J11) settled for about 5 years, and (3) Jengka 2 (J2) settled for about 8 years. The three settlements were geographically separated (Figure 7). After initial briefing of FELDA officials and settler representatives, the survey took place over a 2 day period on each scheme. The intention was to contact as many settlers as possible during that time.

Each settler was interviewed to ascertain basic socio-demographic data, and a blood sample was taken for serological testing. One settler in 5 (all those whose survey identification number ended in 0 or 5) was interviewed further to determine work patterns both on and off the settlement. The social, demographic, and work data is still being analyzed. Within 6 hours following collection of blood, sera were separated and frozen at -20°C until tested. Serological examination included screening for R. tsutsugamushi antibody at a dilution of 1:50 by the indirect FAT (17).

R. tsutsugamushi attack rates were calculated from antibody prevalence rates by methods previously described using a standard annual reversion rate of 0.61 (19). Rates were compared using

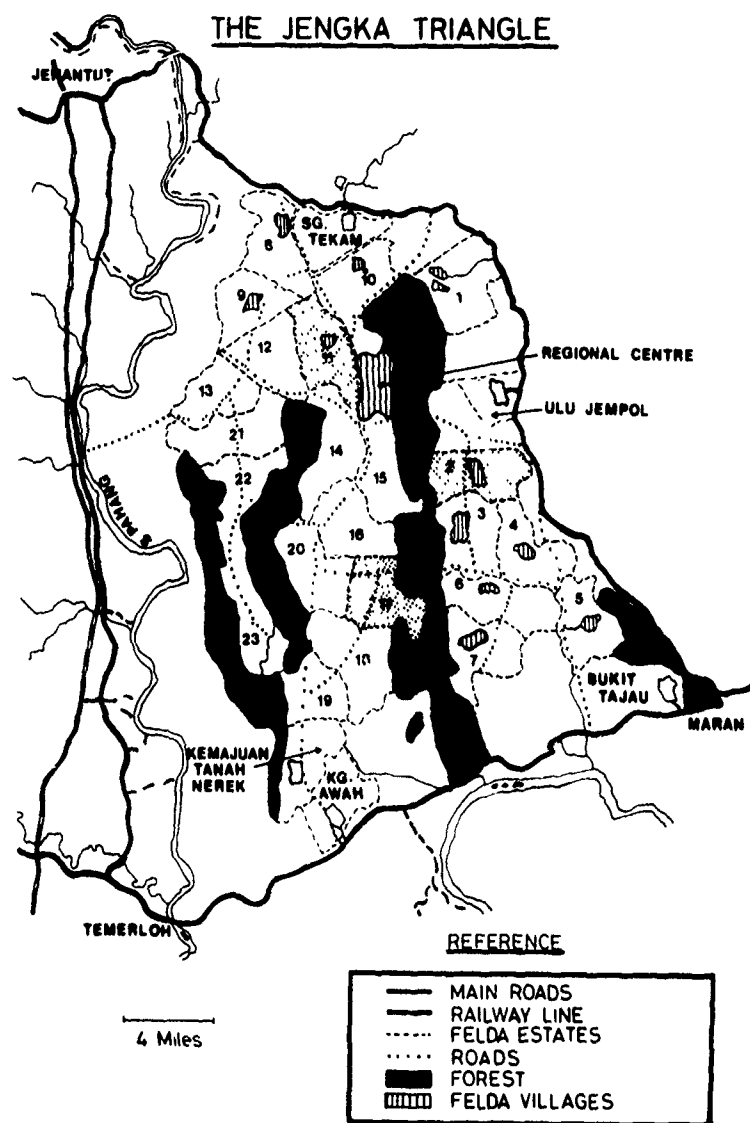


Figure 7. Location of schemes studied.

Chi-square (χ^2), the student's t-test, and curve fitting by trial and error.

Overall compliance of settlers on the 3 schemes was 43.4% (range 32.1% - 63.8%) in the 2 days. Population details, response and antibody prevalence are summarized in Table 4. The distribution of years settled is shown at Figure 8 for each scheme; note that the vast majority of settlers fall within a 1-year band specific for each scheme. In the case of J2, a small group of 14 settlers were 3 years behind the earliest settlers in the main group and, thus, have a disproportionate effect on the mean. They have been excluded from subsequent calculations.

Table 4. Settler response and antibody prevalence by scheme

Scheme	Settler Population	Responders	Per Cent Compliance	Antibody Prevalence/100 (Mean years settled)
J17	398	254	63.8	28.74 (1.16)
J11	471	151	32.1	39.71 (4.75)
J2	373	121	32.4	12.15 (7.43)
Total	1242	526	42.4	28.71

The prevalence rates of antibody were 12% on J2, 40% on J11, and 29% on J17. They differed significantly in the 3 schemes ($\chi^2 = 23.39$, Df = 2, $p < 0.0005$), and each differed significantly from the others (J17/J11, $p < 0.05$; J17/J2 and J11/J2, $p < 0.001$).

The antibody prevalence rates were converted to estimates of attack rates, and the mean time settled on each scheme was adjusted to allow for the interval between planting and settlement. Using functions of the estimated attack rate (\hat{A}) as the ordinate and functions of time planted in years (T) on the same scheme as the abscissa, the data were found to have a statistically significant relationship expressed by the formula:

$$\hat{A} = \frac{1}{2} \log_e \frac{(1 + 0.422 \cdot e^{b \cdot T})}{(1 - 0.422 \cdot e^{b \cdot T})}$$

$$\text{where: } b = (-7.204 \times 10^{-5})$$

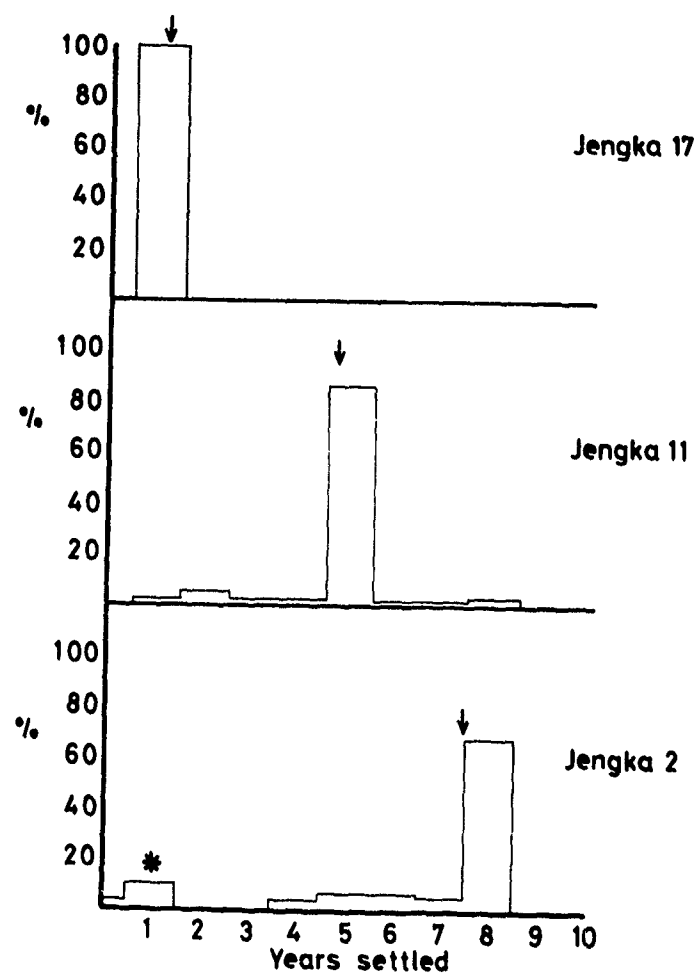


Figure 8. Distribution of scheme populations by time settled.

↓ indicates mean

* excluded from mean (see text)

This curve is shown in Figure 9 with the three points calculated from observation of prevalence indicated.

By substitution of \hat{A} in the equation of Muench (12), estimates of antibody prevalence (\hat{P}) are obtained over the same range of years settled:

$$\hat{P} = \frac{\hat{A}}{\hat{A} + 0.61} (1 - e^{-T_1(\hat{A} + 0.61)})$$

where: T_1 = mean time settled

The resultant curve is shown at Figure 10 with the three observed prevalence rates indicated.

Using the estimate of annual attack rate and correcting for an assumed Poisson distribution, the number of individuals expected to be infected annually can be calculated as a proportion of the population for each point of the curve. Using the 2.3 ratio of infected to sick persons found in Taiwan (13), the number of persons expected to suffer scrub typhus annually can also be calculated as a proportion of the population. These lines are shown in Figure 11. The estimate of morbidity is shown with ± 2 standard errors based on the average number of persons surveyed per scheme.

This study confirms the earlier suggestion that rates of human infection with *R. tsutsugamushi* might vary in oil palm of differing ages. In oil palm developments the antibody prevalence rates can be explained in terms of an initially high attack rate that remains fairly static for about the first 7 years after planting and then declines exponentially to zero in the following 4 to 5 years.

LEPTOTROMBIDIUM (LEPTOTROMBIDIUM) VIVERICOLA, VERCAMMON-GRANDJEAN
AND LANGSTON, 1976, AS A POSSIBLE VECTOR OF SCRUB TYPHUS
IN PENINSULAR MALAYSIA

Three species of *Leptotrombidium* are known to be vectors of scrub typhus in Peninsular Malaysia. These include: *L. (L.) fletcheri* (formerly *akamushi* in Malaysia), *L. (L.) deliense*, and *L. (L.) arenicola*.

L. (L.) fletcheri is found in lalang fields (*Imperata cylindrica*) (10) throughout Peninsular Malaysia. The natural transmission of *R. tsutsugamushi* has been studied within an infected, laboratory-reared colony of *L. (L.) fletcheri* (15,16,29).

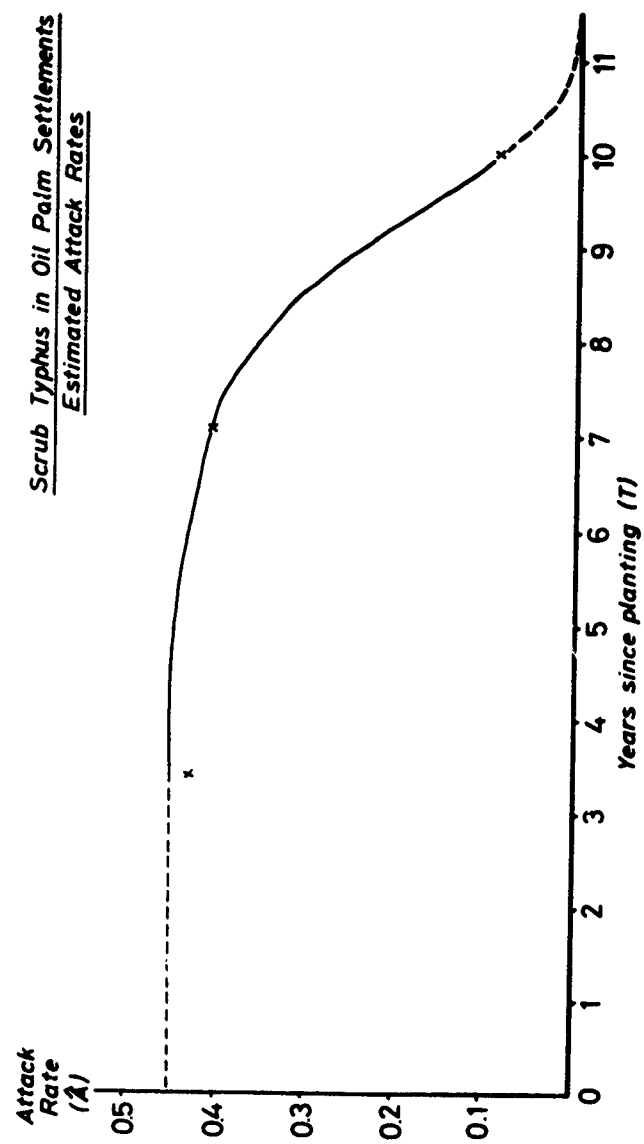


Figure 9. Scrub typhus in oil palm settlements-estimated attack rates.

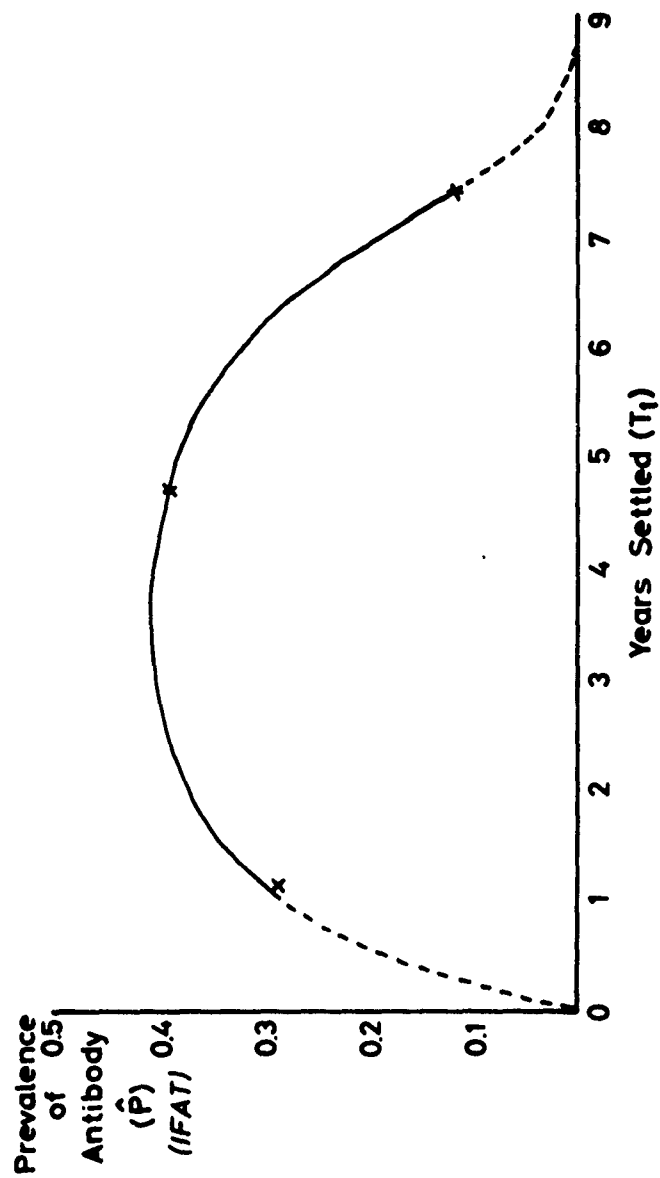


Figure 10. Estimate of antibody prevalence in settlers by years settled.

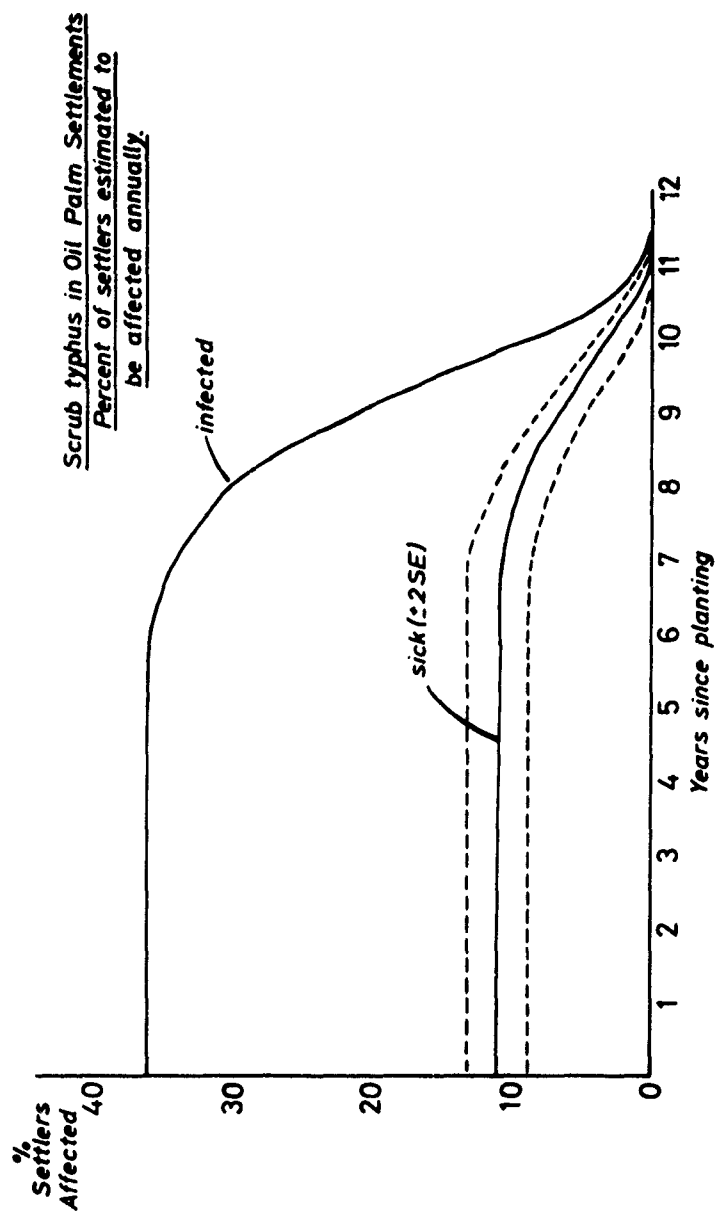


Figure 11. Scrub typhus in oil palm settlements - percent of settlers estimated to be affected annually.

L. (L.) deliense is distributed throughout much of Southeast Asia and is the major vector in forest, scrub, and oil palm habitats throughout Peninsular Malaysia (10). L. (L.) arenicola is found along the sandy beaches of Peninsular Malaysia (26, 27) and has been reported from beaches around Jakarta, Indonesia (9). Transovarial transmission has been reported from 2 separate laboratory-reared colonies of this species (14, 18).

In 1976, a species of chigger was collected that was taxonomically similar to L. (L.) arenicola but was found in inland locations not associated with sandy beaches. Additional specimens have since been collected from various study sites. Recently, Vercammen-Grandjean and Langston (28) described a species, L. (L.) vivericola, which taxonomically conformed to our specimens. Confusion in the taxonomic position of this species has been resolved by obtaining the holotype of the species. The holotype was remeasured, and the discrepancies in the original reported description have been noted. The new measurements along with the averages of a series of collections are presented in Table 5.

In recent years, small collections of L. (L.) vivericola have been made from various study sites, including Elmina Estate (21) Bukit Mendi, and the Jengka Triangle (WRAIR Annual Progress Report, 1 Oct 77 - 30 Sep 78). Infected chiggers have been identified from all of these collection sites. In the Jengka study, over 26% of the total chiggers collected from black plates have been L. (L.) vivericola. However, less than 1% of chiggers collected from mammals have been this species. The majority of the previous collections of L. (L.) vivericola have been from the tree shrew, Tupaia glis. Because few T. glis have been collected from the Jengka study site, ground birds are being investigated as possible major hosts for this species.

TROMBICULID MITE COLONIES

Two colonies of R. tsutsugamushi infected vector chiggers are maintained by the Department of Acarology. The infected colony of L. (L.) fletcheri, currently in its 31st generation, was collected from a lalang field near Kampung Jenderam, Peninsular Malaysia. The L. (L.) arenicola infected colony was collected under vegetation along a sandy beach near Mersing, Peninsular Malaysia and is in its 18th generation.

The transovarial and transtadial infections of both colonies have been studied in detail (15, 18). Additionally, these colonies and their uninfected counterparts have been used in studies on R. tsutsugamushi transmission and on chigger life

Table 5. Scutal measurements of Leptotrombidium (Leptotrombidium) vivericola

Collection Data		Scutal Measurements										
Location	Type & (Number)	AW	PW	SB	ASB	PSB	SD	A-P	AM	AL	PL	Sens
Jengka, Pahang	BP (24)	50.6	59.2	25.5	21	11.1	32.1	24	42.7	34.5	44.4	62.5
Jengka, Pahang	Birds (6)	51.3	59	24.8	20.8	11.1	31.9	23.5	43.2	34.7	44	-
Jengka, Pahang	Rodent (2)	51.5	62	26	21.5	11.5	33	24.5	45	32.5	45	-
Bukit Mendi, Pahang	BP (22)	50.8	58.7	25	21.7	11.5	33.3	24.1	41.6	34.5	45	60.7
Minyah Beku, Johore	BP (5)	49	57.2	23.6	21.4	10.8	32.2	24	40.8	33	41.4	60.7
Ulu Langat, Selangor	Rodent (3)	49.3	60	25	22	11	33.0	24	43.3	35.3	45.7	57
Subang Forest, Selangor	BP (1)	52	59	27	20	10	30	23	41	31	46	-
Holotype*	Rodent (1)	52	59	22	25	12	37	38	50	42	47	70

*Holotype remeasured by IMR Acarology Division, varies slightly from original description of Vercammen-Grandjean and Langston (28).

Abbreviations: AW = Anterior Width; PW = Posterior Width; SB = Sensillae Bases; ASB = Anterior Margin to SB; PSB = Posterior Margin to SB; SD = ASB + PSB; A-P = distance between AL and PL;

AM = Anterior Median Seta; AL = Anteriolateral Setae; PL = Posteriolateral Setae;

Sens = Sensillae.

cycles (5, 16, 29). Characterization of R. tsutsugamushi organisms existing in 22 lines of the 25th generation of the L. (L.) fletcheri colony and 23 lines of the 13th generation of the L. (L.) arenicola colony have been conducted (WRAIR Annual Progress Report, 1 Oct 77 - 30 Sep 78). Chiggers from both the infected and uninfected colonies are supplied to WRAIR for laboratory studies as needed.

During the past reporting period, both infected colonies were used to transmit R. tsutsugamushi to human volunteers in the initial phase of a chemoprophylaxis study (see: Chemoprophylaxis of Laboratory Induced Scrub Typhus with Oral Doxycycline). This was the first time that these colonies have been experimentally fed on humans, and chiggers from both colonies proved highly effective in the transmission of R. tsutsugamushi infections. The L. (L.) fletcheri chiggers appeared to transmit the more virulent organisms and will be utilized in the second phase of this study during the upcoming reporting period.

DISTRIBUTION OF ANTIGENIC STRAINS OF RICKETTSIA TSUTSUGAMUSHI IN ENDEMIC REGION

Background: Antigenic heterogeneity of R. tsutsugamushi has been reported sufficiently for the trait to become widely accepted as characteristic of the organism. Antigenic characterization of many isolates from Thailand, West Pakistan, and Peninsular Malaysia have demonstrated the predominance of Karp and Karp-related strains (7, 20, 22). Knowledge of the prevalence and distribution of antigenic strains of R. tsutsugamushi in the endemic region is an obvious prerequisite to the selection of antigens in the development of immunoprophylactic agents. Isolates from different geographic areas within Peninsular Malaysia and specimens from other areas in the Asiatic-Pacific region were obtained for analysis.

Results:

I. Peninsular Malaysia

A principal Unit study site has been in Central Pahang. Since the findings in this area might not reflect the prevalence and distribution of antigenic types in the entire endemic region, infected chiggers collected from varied habitats throughout Peninsular Malaysia were antigenically analyzed (Table 6). The chiggers were collected by the black-plate technique (8), and the identification of R. tsutsugamushi organisms in unengorged chiggers was made by direct FAT (4) (Table 7). Results showed that 103 of 155 (66%) isolates were monotypic, and the majority of these, 96, were Karp. A tabulation of the frequency of which each

Table 6. Sites of chigger collection in Peninsular Malaysia

State	Location	Habitats	Time
Johore	Kluang, Peletong, Sedeli Kechil, Masai, Minyak Beku	Scrub, lalang, forest	November 1978
Pahang	Bukit Mendi	Oil palm	Mar 76 - Jul 76
Pahang	Cameron Highlands	Highland forest	April 1979
Selangor	Rawang, Jenderam, Kuala Selangor Road, Subang, Bangi, Ulu Langat	Lalang, forest	Apr - May 1978

Table 7. Antigenic characterization of Rickettsia tsutsugamushi organisms found in chiggers collected in Peninsular Malaysia

Antigenic Combinations	Locality				Total
	Johore	Bukit Mendi	Cameron Highlands	Selangor	
Karp	8	70	6	12	96 (61.9) *
TA763	-	6	-	1	7 (4.5)
Karp, Kato	-	2	-	-	2 (1.3)
Karp, TA686	2	2	1	-	5 (3.2)
Karp, TA763	-	1	-	-	1 (0.7)
TA686, TA716	-	-	1	-	1 (0.7)
TA716, TA763	-	1	1	-	2 (1.2)
Karp, TA686, TA716	5	2	1	-	8 (5.2)
Karp, TA686, TA763	2	-	1	-	3 (1.9)
Karp, TA716, TA763	-	1	2	-	3 (1.9)
Karp, TA686, TA716, TA763	4	15	4	-	23 (14.8)
Kato, TA686, TA716, TA763	-	-	1	-	1 (0.7)
Karp, Kato, TA686, TA716, TA763	-	-	3	-	3 (1.9)
TOTAL	21 (13.6)	100 (64.5)	21 (13.6)	13 (8.4)	155 (99.9)

*Number of isolates (%)

(
prototype strain of R. tsutsugamushi occurs among the isolates reveals the overwhelming predominance of the Karp strain (92.9%) (Table 8). The other strains found in these chiggers were TA686, TA763, TA716, and Kato; all are serologically related to Karp (6, 20). With the exception of 5 Babiania parvifera chiggers collected in Selangor, all chiggers belonged to the Leptotrombidium (Leptotrombidium) group (Table 9). This study represents the first confirmed report of infected L. (L.) bodense, L. (L.) keukenschrijveri, and L. (L.) scutellare in Malaysia.

Isolations of scrub typhus organisms from blood of different animals trapped at Bukit Mendi and Johore were attempted. Of those collected at Bukit Mendi, 59 of 312 (18.9%) animals were rickettsemic (WRAIR Annual Progress Report, 1 Oct 77 - 30 Sep 78). Twenty-one rodents trapped in Johore were also tested, but none were found to be infected with R. tsutsugamushi organisms.

II. Thailand

This study represents a cooperative effort with the Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. Unengorged chiggers were collected from various sites throughout Thailand (Figure 12) and shipped in vials of water to Kuala Lumpur for identification of the species and antigenic characterization of R. tsutsugamushi in the infected chiggers. The collections were made intermittently from July 1977 through December 1978.

Table 10 summarizes the species of chiggers collected from the respective sites. These included not only those chiggers examined for the presence of scrub typhus organisms, but also the ones which were dead upon arrival. Leptotrombidium (L.) species comprised 94.4% of entire collection, and L. (L.) deliense was the most predominant of these (61.5%).

The infection rates in the chiggers obtained from 4 of 7 study sites were as follows: Chiang Mai - 47/524 (9.0%), Nakorn Ratchasima - 52/382 (13.6%), Pak Chong - 21/297 (7.1%), Ubon Ratchathani - 26/675 (3.9%). The chiggers from the other 3 areas were not examined due to technical problems. Species of infected chiggers are recorded in Table 11. Ninety-five percent of the infected chiggers belonged to the Leptotrombidium (L.) group, and L. (L.) deliense comprised the largest single group of infected chiggers (59.6%). This is the first recorded incidence of infected chigger species other than L. (L.) deliense in Thailand. The role that infected non-trombiculid mites might play in the epidemiology of scrub typhus is not known.

Results of typing the R. tsutsugamushi organisms in the infected chiggers are shown in Tables 12 and 13. Monotypic infections were observed in 76.7% of the chiggers. There appears

Table 8. Frequency of Rickettsia tsutsugamushi prototype antigens found among the chiggers collected in Peninsular Malaysia

Prototype	Locality								Total	
	Johore		Bukit Mendi		Cameron Highlands		Selangor		Number	Frequency
	Number	Frequency*	Number	Frequency	Number	Frequency	Number	Frequency		
Karp	21	100.0	93	93.0	18	85.7	12	92.3	144	92.9
TA686	13	61.9	19	19.0	12	57.1	-	-	44	28.4
TA763	6	28.6	24	24.0	12	57.1	1	7.7	43	27.7
TA716	9	42.9	19	19.0	13	61.9	-	-	41	26.5
Kato	-	-	2	2.0	4	19.1	-	-	6	3.9
Gilliam	-	-	-	-	-	-	-	-	-	-
TA678	-	-	-	-	-	-	-	-	-	-
TH1817	-	-	-	-	-	-	-	-	-	-

*Expressed as the percentage of chiggers in which the respective antigen was identified.

Table 9. Infection rate of chiggers collection in Peninsular Malaysia

Chigger Species	Locality				Total
	Johore	Bukit Mendi	Cameron Highlands	Selangor	
<u>Leptotrombidium</u> (<u>Leptotrombidium</u>) <u>bodense</u>	2/21	-	0/47	0/9	2/77 (2.6)*
<u>Leptotrombidium</u> (<u>Leptotrombidium</u>) <u>deliense</u>	18/288	85/1776	-	0/74	103/2138 (4.8)
<u>Leptotrombidium</u> (<u>Leptotrombidium</u>) <u>fletcheri</u>	1/13	-	-	13/391	14/404 (3.5)
<u>Leptotrombidium</u> (<u>Leptotrombidium</u>) <u>keukenschrijveri</u>	-	-	15/646	-	15/646 (2.3)
<u>Leptotrombidium</u> (<u>Leptotrombidium</u>) <u>scutellare</u>	-	-	6/235	-	6/235 (2.6)
<u>Leptotrombidium</u> (<u>Leptotrombidium</u>) <u>vivericola</u>	0/5	15/359	0/53	0/371	15/788 (1.9)
<u>Babangia</u> <u>parmifera</u>	-	-	-	0/5	0/5 (0)
TOTAL	21/327 (6.4)	100/2135 (4.7)	21/981 (2.1)	13/850 (1.5)	155/4293 (3.6)

*Number of positive chiggers/number of total chiggers (% infected)



Figure 12. Locations of black-plated chigger collection sites in Thailand. (1) Chiang Mai, (2) Nakorn Ratchasima, (3) Ubon Ratchathani, (4) Surin, (5) Kanchanaburi, (6) Prachin Buri, (7) Pak Chong.

Table 10. Chigger species collected from various sites throughout Thailand

LOCATION	<i>L. (L.) deliense</i>	<i>L. (L.) fulleri</i>	<i>L. (L.) miculum arivum</i>	<i>L. (L.) scanloni</i>	<i>L. (L.) scutellare</i>	<i>L. (L.) sp. A</i>	<i>L. (L.) sp. B</i>	<i>L. (L.) sp. C</i>	<i>L. (L.) sp. D</i>	<i>L. (L.) sp. G</i>	<i>L. (L.) paniculatum</i>	<i>Ascoschoengastia indica</i>	<i>Babiania parmitera</i>	<i>Eutrombicula wichmanni</i>	<i>Gahriepia sp. A</i>	<i>Helicula sp.</i>	<i>Microtrombicula chamlongi</i>	<i>Odonacarus sp.</i>	<i>Siseca rara</i>	<i>Walchiella oudemansi</i>	<i>Walchiella traubi</i>	TOTAL
Chiang Mai	41	1	123	1	392	119	4	-	1	1	-	-	-	-	-	-	-	-	-	-	-	683
Kanchanaburi	91	-	3	-	-	-	-	-	1	-	1	-	-	-	-	-	-	-	-	-	-	96
Nakhorn Ratchasima	635	21	19	-	374	-	-	-	2	-	61	-	-	-	20	13	5	-	16	70	1	1237
Pak Chong	424	-	63	-	-	-	-	2	1	-	6	2	1	-	-	7	-	75	-	-	-	581
Prachin Buri	-	-	-	-	-	-	-	-	-	-	9	-	-	-	-	-	-	-	-	-	-	9
Surin	145	-	-	-	-	-	-	15	-	-	-	-	-	-	2	-	-	-	-	-	-	162
Ubon Ratchathani	949	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	950
TOTAL NUMBER	2285	22	208	1	766	119	4	17	5	1	77	2	1	20	2	20	6	75	16	70	1	3718
% of TOTAL	61.5	0.6	5.6	0.03	20.6	3.2	0.1	0.5	0.1	0.03	2.1	0.05	0.03	0.5	0.03	0.5	0.2	2.0	0.4	1.9	0.03	100

Table 11. Chigger species infected with Rickettsia tsutsugamushi from Thailand

LOCATION	<u>L. (L.) delense</u>	<u>L. (L.) miculum arvinum</u>	<u>L. (L.) scutellare</u>	<u>L. (L.) sp. A</u>	<u>L. (L.) sp. B</u>	<u>L. (L.) paniculatum</u>	<u>Eutrombicula wichmanni</u>	<u>Microtrombicula chamlongi</u>	<u>Odontacarus sp.</u>	TOTAL
Chiang Mai	15	6	17	7	2	-	-	-	-	47
Nakorn Ratchasima	31	-	17	-	-	1	2	1	-	52
Pak Chong	15	3	-	-	-	-	-	-	3	21
Ubon Ratchathani	26	-	-	-	-	-	-	-	-	26
TOTAL	87(59.6)*	9(6.2)	34(23.3)	7(4.8)	2(1.4)	1(0.7)	2(1.4)	1(0.7)	3(2.1)	146(100.2)

*Number of infected chiggers (%)

Table 12. Antigenic characterization of Rickettsia tsutsugamushi organisms found in chiggers collected in Thailand

Antigenic Combinations	Locality				Total
	Chiang Mai	Nakorn Ratchasima	Pak Chong	Ubon Ratchathani	
Karp	25	22	19	8	74 (50.7)*
Kato	-	1	1	-	2 (1.4)
TA686	1	1	1	3	6 (4.1)
TA716	7	-	-	6	13 (8.9)
TA763	9	7	-	1	17 (11.6)
Karp, TA716	1	2	-	1	4 (2.7)
Karp, TA763	1	3	-	-	4 (2.7)
Karp, TA716, TA763	1	3	-	-	4 (2.7)
Karp, TA686, TA716	-	-	-	2	2 (1.4)
TA716, TA763	2	12	-	-	14 (9.6)
TA686, TA716	-	-	-	4	4 (2.7)
TA686, TA763	-	-	-	1	1 (0.7)
Kato, TA716, TA763	-	1	-	-	1 (0.7)
TOTAL	47 (32.2)	52 (35.6)	21 (14.4)	26 (17.8)	146 (99.9)

*Number of isolates (%)

Table 13. Antigenic characterization of *Rickettsia tsutsugamushi* organisms found in various infected chigger species.

ANTIGENIC COMBINATIONS	L. (L.) <i>deliense</i>	L. (L.) <i>miculum arvinum</i>	L. (L.) <i>scutellare</i>	L. (L.) sp. A	L. (L.) sp. B	L. (L.) <i>paniculatum</i>	<i>Eutrombicula wichmanni</i>	<i>Microtrombicula chamlongi</i>	<i>Odontacarus</i> sp.	TOTAL
Karp	30	4	34	-	-	1	2	-	3	74 (50.7)*
Kato	1	-	-	-	-	-	-	1	-	2 (1.4)
TA686	5	-	-	1	-	-	-	-	-	6 (4.1)
TA716	7	2	-	3	1	-	-	-	-	13 (8.9)
TA763	12	1	-	3	1	-	-	-	-	17 (11.6)
Karp, TA716	3	1	-	-	-	-	-	-	-	4 (2.7)
Karp, TA763	4	-	-	-	-	-	-	-	-	4 (2.7)
Karp, TA716, TA763	4	-	-	-	-	-	-	-	-	4 (2.7)
Karp, TA686, TA716	2	-	-	-	-	-	-	-	-	2 (1.4)
TA716, TA763	13	1	-	-	-	-	-	-	-	14 (9.6)
TA686, TA716	4	-	-	-	-	-	-	-	-	4 (2.7)
TA686, TA763	1	-	-	-	-	-	-	-	-	1 (0.7)
Kato, TA716, TA763	1	-	-	-	-	-	-	-	-	1 (0.7)
TOTAL	87 (59.6)	9 (6.2)	54 (23.3)	7 (4.8)	2 (1.4)	1 (0.7)	2 (1.4)	1 (0.7)	3 (2.1)	146 (99.9)

*Number of isolates (%)

to be no correlation between either the antigens and the collection sites or the antigens and the chigger species. Table 14 summarizes the frequency in which the prototype R. tsutsugamushi strains occur among the infected chiggers. The most common strain was Karp (60.3%), and the only other strains found were the Karp-related ones (TA716, TA763, TA686 and Kato).

III. Taiwan

Isolates from humans, rodents, and chiggers were provided by Dr. James Coolbaugh, formerly of Naval Medical Research Unit 2, Taiwan. Rodent isolations were made from either single or pooled rat spleen/kidney suspensions. Pools of chiggers were used for isolating R. tsutsugamushi organisms from vectors. The isolates were characterized antigenically by the direct FAT (Table 15). Results showed that 26 of 49 (53.1%) isolates contained mixtures of 2 or more strains. TA716 and Karp strains were the most frequently occurring antigens among the isolates (Table 16). Gilliam and TA678 strains were not identified.

IV. Philippines Islands

Rodent isolates were provided by Dr. James Coolbaugh. The direct FAT was used to characterize the isolates antigenically (Table 17). Two or more antigens were found in 87.8% of the isolates, and the most predominant antigens in order of frequency were TA716, TA686, Karp, and TA763 (Table 18).

V. Banks Islands and Ndende Island

Rats and chiggers were collected in the Banks Islands, Northern New Hebrides, and in different sites of Ndende Island in the Santa Cruz group. The isolates were provided by Dr. J.A.R. Miles of University of Otago, Dunedin, New Zealand, and were characterized antigenically by the direct FAT (Table 19). A majority of the isolates (72.7%) contained mixtures of 2 or more antigens. The most common antigen was TA716, with the remaining being Karp, TA763 and TA686 (Table 20).

VI. Australia

Fifty-two isolates from humans, rodents, and chiggers were provided by Mr. Ray Campbell of the Queensland Institute for Medical Research, Brisbane, Queensland. Direct FAT was used to characterize the isolates antigenically (Table 21). Two or more antigens were present in 71.2% of the isolates. TA716 antigen was the most prevalent (94.2%) antigen, followed by TA763, TA686, and Karp antigens (Table 22). Gilliam antigen was present in 2 rodent isolates, one in combination with TA716 and the other being monotypic.

Table 14. Frequency of Rickettsia tsutsugamushi prototype antigens found among the chiggers collected in Thailand

Prototype Antigens	Locality										Total	
	Chiang Mai		Nakhorn Ratchasima		Pak Chong		Ubon Ratchathani					
	Number	Frequency*	Number	Frequency	Number	Frequency	Number	Frequency	Number	Frequency	Number	Frequency
Karp	28	59.6	30	57.7	19	90.5	11	42.3	88	60.3		
TA716	11	23.4	18	34.6	-	-	13	50.0	42	28.8		
TA763	13	27.7	26	50.0	-	-	2	7.7	41	28.1		
TA686	1	2.1	1	1.9	1	4.8	10	38.5	13	8.9		
Kato	-	-	2	3.8	1	4.8	-	-	3	2.1		
Gilliam	-	-	-	-	-	-	-	-	-	-		
TA678	-	-	-	-	-	-	-	-	-	-		
TH1817	-	-	-	-	-	-	-	-	-	-		

*Expressed as the percentage of chiggers in which the respective antigen was identified.

Table 15. Antigenic characterization of Rickettsia tsutsugamushi organisms isolated from humans, rodents and chiggers in Taiwan

Antigenic Combinations	Isolates			Total
	Human	Rodent	Chigger	
TA716	12	8	3	23 (46.9) *
Karp, TA716	3	7	7	17 (34.7)
TA716, TA763	1	-	-	1 (2.0)
TA716, TH1817	-	1	1	2 (4.1)
Karp, TA716, TA763	1	-	3	4 (8.2)
Karp, TA686, TA716	-	1	-	1 (2.0)
Karp, TA686, TA716, TA763	-	-	1	1 (2.0)
TOTAL	17 (34.7)	17 (34.7)	15 (30.6)	49 (99.9)

*Number of isolates (%)

Table 16. Frequency of Rickettsia tsutsugamushi prototype antigens found among the isolates collected in Taiwan

Prototype Antigens	Isolates						Total	
	Human		Rodent		Chigger		Number	Frequency
TA716	Number	Frequency*	Number	Frequency	Number	Frequency	Number	Frequency
	17	73.9	17	63.0	15	46.9	49	59.8
Karp	4	17.4	8	29.6	11	34.4	23	28.0
TA763	2	8.4	-	-	4	12.5	6	7.3
TA686	-	-	1	3.7	1	3.1	2	2.4
TH1817	-	-	1	3.7	1	3.1	2	2.4
Kato	-	-	-	-	-	-	-	-
Gilliam	-	-	-	-	-	-	-	-
TA678	-	-	-	-	-	-	-	-

*Expressed as the percentage of isolates in which the respective antigen was identified.

Table 17. Antigenic characterization of Rickettsia
tsutsugamushi organisms isolated from
rodents in Philippines

Antigenic Combinations	Number (%)
Karp	1 (2.4)
TA686	1 (2.4)
TA716	2 (4.9)
TA763	1 (2.4)
Karp, TA686	1 (2.4)
Karp, TA716	1 (2.4)
Karp, TA763	1 (2.4)
TA686, TA716	5 (12.2)
TA716, TA763	1 (2.4)
Karp, TA686, TA716	5 (12.2)
Karp, TA686, TA763	1 (2.4)
Karp, TA716, TA763	1 (2.4)
TA686, TA716, TA763	4 (9.8)
Karp, TA686, TA716, TA763	15 (36.6)
Karp, Kato, TA686, TA716, TA763	1 (2.4)
TOTAL	41 (99.7)

Table 18. Frequency of Rickettsia
tsutsugamushi prototype antigen
found among the rodent isolates
collected in Philippines

Prototype Antigen	Isolates	
	Number	Frequency*
TA716	35	28.9
TA686	33	27.3
Karp	27	22.3
TA763	25	20.7
Kato	1	0.8
Gilliam	-	-
TA678	-	-
TH1817	-	-

*Expressed as the percentage of isolates
in which the respective antigen was
identified.

Table 19. Antigenic characterization of Rickettsia tsutsugamushi organisms isolated from rodents and chiggers collected in Banks and Ndende Islands

Antigenic Combinations	Isolates		Total
	Rodents	Chiggers	
TA716	3	-	3 (27.3)*
Karp, TA716	-	1	1 (9.1)
TA716, TA763	1	-	1 (9.1)
Karp, TA716, TA763	2	-	2 (18.2)
Karp, TA686, TA716, TA763	1	3	4 (36.4)
TOTAL	7 (63.6)	4 (36.4)	11 (100.1)

*Number of isolates (%)

Table 20. Frequency of Rickettsia tsutsugamushi prototype antigens found among the isolates collected in Banks and Ndende Islands

Prototype Antigen	Isolates				Total	
	Rodents		Chiggers			
	Number	Frequency*	Number	Frequency	Number	Frequency
TA716	7	46.7	4	28.6	11	37.9
Karp	3	20.0	4	28.6	7	24.1
TA763	4	26.7	3	21.4	7	24.1
TA686	1	6.7	3	21.4	4	13.8
Kato	-	-	-	-	-	-
Gilliam	-	-	-	-	-	-
TA678	-	-	-	-	-	-
TH1817	-	-	-	-	-	-

*Expressed as the percentage of isolates in which the respective antigen was identified

Table 21 Antigenic characterization of Rickettsia tsutsugamushi organisms isolated from humans, rodents and chiggers in Australia

Antigenic Combinations	Isolates			Total
	Human	Rodent	Chigger	
Gilliam	-	1	-	1 (1.9)*
TA686	-	2	-	2 (3.8)
TA716	5	3	4	12 (23.1)
Karp, TA716	4	2	-	6 (11.5)
Gilliam, TA716	-	1	-	1 (1.9)
TA686, TA716	4	1	-	5 (9.6)
TA716, TA763	6	3	-	9 (17.3)
Karp, TA686, TA716	-	3	-	3 (5.8)
Karp, TA716, TA763	4	2	-	6 (11.5)
TA686, TA716, TA763	3	3	-	6 (11.5)
Karp, TA686, TA716, TA763	1	-	-	1 (1.9)
TOTAL	27 (51.9)	21 (40.4)	4 (7.7)	52 (99.8)

*Number of isolates (%)

Table 22. Frequency of Rickettsia tsutsugamushi prototype antigens found among the isolates collected in Australia

Prototype Antigens	Isolates								Total	
	Human		Rodent		Chigger					
	Number	Frequency*	Number	Frequency	Number	Frequency	Number	Frequency	Number	Frequency
TA716	27	100.0	18	85.7	4	100.0	49	94.2		
TA763	14	51.9	8	38.1	-	-	22	42.3		
TA686	8	29.6	9	42.9	-	-	17	32.7		
Karp	9	33.3	7	33.3	-	-	16	30.8		
Gilliam	-	-	2	9.5	-	-	2	3.8		
Kato	-	-	-	-	-	-	-	-		
TA678	-	-	-	-	-	-	-	-		
TH1817	-	-	-	-	-	-	-	-		

*Expressed as the percentage of isolates in which the respective antigen was identified.

VII. Indonesia

Isolation attempts were made on frozen blood from 131 rodents, provided by Dr. Lim Boo Liat, WHO/Vector Biology and Control Research Unit No.2, Jakarta, Indonesia. R. tsutsugamushi organisms were not found in any of the rodent blood.

Summary:

1. Consolidating the data from the 7 countries, 54.7% of the total isolates contained only a single antigen, while 45.3% had mixtures of 2 or more antigens (Table 23). Taken separately, chigger isolates were more monotypic (68.5%) than multitypic (31.5%).
2. The predominant antigens throughout the endemic areas were the serologically related TA716, Karp, TA763, and TA686 antigens.

ANTIGENIC COMPARISON OF PROTOTYPE AND FIELD ISOLATES

The identification and the characterization of the most common strain(s) in a given area is required for the selection of a proper combination of strains to comprise an effective vaccine. The existence of multiple antigenic strains in Peninsular Malaysia has been demonstrated by the direct fluorescent antibody technique. We felt a need to compare the antigenic components of the existing prototype strains, as well as some selected field isolates, by a technique which could more closely be correlated with an organism's ability to protect an animal from R. tsutsugamushi infection. The cross neutralization test was selected for this purpose. Only limited study has been done to date. The basics of the test, such as the optimal volumes of serum and rickettsiae to be used and the proper incubation time and temperature of the test, have been determined. High titered antisera are currently being prepared in rabbits.

STUDIES OF CELL MEDIATED IMMUNITY IN RICKETTSIA TSUTSUGAMUSHI INFECTION

The emphasis of this study has been to develop an adequate scrub typhus antigen to measure the specific responses of the sensitized lymphocytes. Attempts to prepare concentrated antigens and to prepare antigens by different procedures have yielded unsatisfactory results. Despite these setbacks, the development of a cell-mediated immunity (CMI) test for scrub typhus is of the

Table 23. Antigenic composition of human, rodent and chigger isolates collected from 7 different countries

Countries	Isolates							
	Human		Rodent		Chigger		Total	
	1*	≥2**	1	≥2	1	≥2	1	≥2
Malaysia	-	-	15	36	103	52	118	88
Thailand	-	-	-	-	112	34	112	34
Taiwan	12	5	8	9	3	12	23	26
Philippines	-	-	5	36	-	-	5	36
Melanesia	-	-	3	4	-	4	3	8
Australia	5	22	6	15	4	-	15	37
Indonesia	-	-	-	-	-	-	-	-
TOTAL	17(38.6)***	27(61.4)	37(27.0)	100(73.0)	222(68.5)	102(31.5)	276(54.7)	229(45.3)

* Single antigen

** Mixture of 2 or more antigens

*** Number of isolates containing specified number of antigen (%)

utmost importance. More emphasis will be placed on CMI study with the evaluation of other in vitro correlates of CMI, such as the leucocyte adherence inhibition, macrophage migration inhibition, and macrophage rickettsiacidal tests. Special emphasis will be given to the latter test. Attempts to develop a lymphocyte transformation test will also continue.

EXPERIMENTAL RICKETTSIA TSUTSUGAMUSHI INFECTION IN LABORATORY-BRED CYNOMOLGUS MONKEYS, MACACA FASCICULARIS

Background: Previous studies in our laboratory have demonstrated the susceptibility of cynomolgus monkeys, Macaca fascicularis, to R. tsutsugamushi infection (WRAIR Annual Progress Reports, 1 Oct 76 - 30 Sep 77; 1 Oct 77 - 30 Sep 78). These monkeys were wild-caught and free of demonstrable antibodies to R. tsutsugamushi when tested by the indirect immunofluorescent test. However, the possibility of previous natural infection which could alter the animal's response to experimental infection could not be excluded completely. The availability of cynomolgus monkeys bred in the laboratory and maintained under conditions that eliminated exposures to vector chiggers allowed us to compare R. tsutsugamushi infection in laboratory-bred and wild-caught animals.

I. Primary Infection

Graded doses of $10^{6.6}$, $10^{5.6}$, and $10^{4.6}$ 50% mouse infectious doses (MID_{50}) of the Karp strain were administered intradermally (ID) and intravenously (IV) into different groups of monkeys. Clinical, hematological, and serological responses to infection were examined. Rickettsemic studies were also performed.

The responses of the monkeys to ID and IV inoculation of the Karp strain of R. tsutsugamushi are summarized in Table 24. The ID-infected animals receiving the highest dose had the most pronounced reactions. Similar responses were observed in IV-infected monkeys, but no clinical signs were seen in the monkeys receiving $10^{4.6} MID_{50}$. Four deaths occurred in the IV-inoculated animals, 3 in the $10^{6.6} MID_{50}$ inoculated group and one in the $10^{5.6} MID_{50}$ inoculated group. In all cases, the animals became anemic, leucopenic, thrombocytopenic, and demonstrated an increase in erythrocyte sedimentation rate. However, the degree of alteration was directly proportional to the dosage. Generally, the antibody responses to the homologous and heterologous antigens were similar in both ID- and IV-inoculated animals, with the maximum titers (1:200 - 1:400) attained 2 months post-inoculation. The titers were slightly higher in IV-inoculated animals during

Table 24. Responses of laboratory-bred cynomolgus monkeys to intradermal and intravenous inoculation of Karp strain of *Rickettsia tsutsugamushi**

Dose	ID-INFECTED				IV-INFECTED			
	No. with Fever ($\leq 40^{\circ}\text{C}$)	No. with Regional Lymphadenopathy	No. with Eschar	No. with Rickettsemia (Days)**	No. with Fever ($\geq 40^{\circ}\text{C}$)	No. with Other Clinical Signs***	No. with Rickettsemia (Days)**	No. of Death (Day)****
$10^{6.6}$	5	5	5	5 (4-22)	5	5	5 (1-25)	3 (9;11;36)
$10^{5.6}$	5	5	5	5 (4-22)	5	4	5 (1-22)	1 (10)
$10^{4.6}$	3	3	3	3 (4-22)	-	-	2 (1-22)	-

* 5 animals inoculated in groups receiving $10^{6.6}$ and $10^{5.6}$ 50% mouse infectious doses (MID_{50}) ID & IV.

3 animals received $10^{4.6}$ MID_{50} ID and 2 monkeys infected with $10^{4.6}$ MID_{50} IV.

** Range of days post-infection in which rickettsemia was observed.

*** Anorexia, lethargy, weakness.

**** Day post-inoculation on which the animal died.

comparable times. In ID-inoculated monkeys, the heterologous antibodies appeared about a week later and tended to disappear earlier.

II. Secondary Infection

On 125 days post-primary infection, 21 survivors from the above study were placed into 5 groups and were inoculated ID with Karp, Gilliam, TA763, Karp and Gilliam, or Karp and TA763. Animals were examined for the same responses.

Results of the challenge are summarized in Table 25. Karp-infected animals were resistant to homologous challenge, but not to heterologous ones. Even though TA763 is serologically related to Karp, fever and rickettsemia were observed. When the challenge inoculum contained both Karp and TA763, the response was more severe in that regional lymphadenopathy and eschar were observed in 2 of 5 monkeys. In cases where rickettsemia was demonstrated, no Karp organisms were detected. This was true even when the inoculum consisted of Karp organisms. No deaths were noted in any monkeys following challenge.

Summary:

1. The susceptibility of the laboratory-bred cynomolgus monkeys to the Karp strain of R. tsutsugamushi compared favorably to that observed in wild-caught animals.
2. The animals were protected against homologous challenge after 125 days, but not against heterologous challenge, including TA763 strain which is serologically related to Karp.

CHEMOPROPHYLAXIS OF LABORATORY INDUCED SCRUB TYPHUS WITH ORAL DOXYCYCLINE

Background: Currently there are no practical means of preventing scrub typhus. An effective vaccine or safe chemoprophylactic agent is not available. Control of the vector chigger by removing vegetation, controlling rodents, spraying with acaricides, or using repellants is either impractical or ineffective.

Attempts at chemoprophylaxis have been made. Smadel and co-workers (23, 24, 25) found chloramphenicol to be unsuitable even before the occasionally disastrous side effect of bone marrow aplasia was associated with its use. In a more recent study, investigators working in the Pescadores Islands compared the effects of weekly, oral, 200 mg doxycycline with those of a

Table 25. Responses of laboratory-bred, Karp-infected cynomolgus monkeys to intradermal challenge with different strain(s) of Rickettsia tsutsugamushi*

Challenge Strain(s)	No. Challenged	No. with Fever ($\geq 40^{\circ}\text{C}$)	No. with Regional Lymphadenopathy	No. with Eschar	Rickettsemia		
					No.	Day**	Strain***
Karp	4	1	-	-	-	-	-
Gilliam	4	3	4	4	4	4-13	Gilliam
TA763	4	3	-	-	4	4-19	TA763
Karp + Gilliam	4	3	3	4	4	4-16	Gilliam
Karp + TA763	5	1	2	2	5	4-22	TA763

* Animals received $10^{6.7}$ 50% mouse infectious dose (MID_{50}) of Karp, 10^6 MID_{50} of Gilliam, and $10^{4.5}$ of TA763 strains 125 days post-primary infection. In the case where 2 strains were given, equal volumes of the 2 dosages were mixed before inoculating the monkeys.

** Range of days post-challenge in which rickettsemia was observed.

*** Strain(s) of R. tsutsugamushi found in monkey blood.

placebo in a large population of Taiwanese troops (Naval Medical Research Unit 2, Taiwan, personal communication). An acceptable statistical difference was not attained due to the combined effects of a low incidence of scrub typhus and problems of compliance. However, the study did demonstrate the safety of doxycycline. No serious side effects were observed in several hundred soldiers given 200 mg doxycycline weekly for 5 months.

We recently published the results of a single dose doxycycline therapeutic trial for presumptive scrub typhus which showed the efficacy of the drug in therapy and its safety even in a situation confused by multiple and potentially serious febrile illnesses (2). The only side effect of note encountered was gastric irritation, a symptom that was at least aggravated by the anorexia and malaise of febrile patients. The study found no evidence of patient sensitivity or photosensitization despite being conducted in the tropics.

A study to determine the efficacy of a single, 200 mg dose of oral doxycycline given weekly for the prevention of scrub typhus morbidity was approved by the IMR Committee on Research Involving Human Subjects, the WRAIR Human Use Committee, and, in principal, by the Surgeon General's Human Subjects Research Review Board. The latter board withheld final approval of Phase II of the study pending the successful completion of Phase I. Phase I was designed to test the feasibility of using laboratory reared chiggers to infect volunteers in the definitive Phase II portion of the study and was successfully completed this year. The results of Phase I are reported here.

Results: R. tsutsugamushi infected L. (L.) fletcheri chiggers were attached to two volunteers and infected L. (L.) arenicola to one volunteer using capsules (3). The L. (L.) fletcheri chiggers attached readily and rapidly on both subjects. L. (L.) arenicola attached slowly and with difficulty. All attached chiggers engorged and were removed from the subjects approximately 50 hours after application. Engorged chiggers were tested for R. tsutsugamushi infection using the direct fluorescent antibody test (4). All were infected.

All subjects developed typical clinical signs and symptoms of scrub typhus which included fever, eschar formation with regional lymphadenopathy, severe headache, and myalgia. The disease appeared to assume a milder course in the L. (L.) arenicola subject. The onset of symptoms was on day 7 post chigger attachment (PCA) for the two L. (L.) fletcheri subjects and day 9 for the L. (L.) arenicola subject. Only the L. (L.) fletcheri subjects developed a transient generalized rash as a result of infection. Laboratory findings also indicated the one L. (L.) arenicola subject suffered a milder scrub typhus infection. All subjects experienced

a profound depression in platelet counts and marked leucopenia. The only consistent biochemical finding in the 3 subjects was hyperkalemia. Only the 2 L. (L.) fletcheri subjects developed elevated lactic dehydrogenase and serum glutamic oxaloacetic transaminase levels.

Rickettsemias were consistently present from day 10 through day 22 PCA in the L. (L.) fletcheri subjects but were only intermittently observed in the L. (L.) arenicola subject (Table 26). Serum antibody conversions were detected in all volunteers by day 22 PCA using the indirect fluorescent antibody test (Table 27).

All subjects received one dose of 200 mg doxycycline beginning on day 3 of illness and recovered uneventfully.

Table 26. Rickettsias of Phase I volunteers

Day Post Attachment of Chiggers	<u>L. (L.) fletcheri</u>		<u>L. (L.) arenicola</u>
	Subject A	Subject B	
1	-	-	-
4	-	-	-
7	+	-	-
10	+	+	-
13	+	+	+
16	+	+	-
22	+	+	+
25 - 43	-	-	negative through day 34 ^a

^aSubject voluntarily withdrew from study.

Table 27. Indirect fluorescent antibody titers of Phase I
volunteers^a

Day Post Attachment of Chiggers	<u>L. (L.) fletcheri</u>		<u>L. (L.) arenicola</u>
	Subject A	Subject B	Subject A
1	neg	-	neg
7	neg	neg	neg
13	neg	50	neg
19	50	400	50
25	400	800	200
31	200	200	100
37	200	200	ND ^b
43	100	50	ND
49	100	100	ND

^aAll titers expressed as the reciprocal of the last serum
dilution reacting with the antigen.

^bNot done: subject voluntarily withdrew from study.

Project 3M762770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 007 Field Studies of Rickettsioses and Other Tropical Diseases

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY / CLASSIFICATION DA OC 6465		2 DATE OF SUMMARY 79 10 01		3 REPORT CONTRACT NUMBER DD FORM 1498 (AR) 36	
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27 KEYWORDS (Provide with Security Classification Code) (U) Infectious Diseases; (U) Dengue; (U) Hepatitis; (U) Wound Infections; (U) Scrub Typhus; (U) Gonorrhea; (U) Vectors; (U) Diarrhea.									
28 TECHNICAL OBJECTIVE, 29 APPROACH, 30 PROGRESS (Provide individual paragraphs identified by number. Provide last of each with Security Classification Code) 23. (U) To define the ecological and biological factors that predispose U.S. military personnel to tropical infectious diseases. Characterization of disease organisms and response to infections is done in support of vaccine development. 24. (U) The etiology, epidemiology, and ecology of disease organisms are studied in the field and in hospital. In vitro cultivation, serological procedures, microbiological assays, mosquito inoculation, vector colonization, and other techniques are used to characterize disease organisms. 25. (U) 78 10 - 79 09 Dengue, hepatitis (HAV, HBV, HANB), war wound infections, diarrheal diseases, scrub typhus, and gonorrhea were studied. Of 37 dengue isolates made in 1978, 31 were type 2, 1 was type 3, and 5 were type 4. Infections occurred in 5-15 percent of nonimmune children. Autopsies revealed viral antigen predominantly in the reticuloendothelial cells. Dengue virus was recovered from 5 percent of A. aegypti mosquitoes collected. Virus was isolated from patients by intrathoracic inoculation of mosquitoes. Virtually 100 percent of Thai adults had antibody to HAV. HAV accounted for 75 percent of cases of acute hepatitis in young children, 25 percent in young adults, and less than 10 percent in adults over 30 years. HANB has been found to be most common in adults. Studies of war wounds showed a prevalence of lower extremity injury and infections mostly involving Pseudomonas and enteric bacteria. Diarrheal diseases in Americans new to the Asian environment were studied. Nine chigger species were found to harbor the scrub typhus rickettsia. The infection rate was 8 percent. A ELISA test was developed for diagnosis of scrub typhus. Among penicillin-resistant N. gonorrhoeae, 18.5 percent produced penicillinase. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.									

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Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 008 Tropical and Subtropical Diseases in Military
Medicine

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1. Etiology of Pediatric Diarrhea at Children's Hospital, Bangkok

OBJECTIVE: To determine the etiology of diarrhea in children seen at Children's Hospital.

BACKGROUND: Enteric infections are a serious cause of morbidity and mortality in developing, tropical countries. Salmonella, Shigella and Vibrio have previously been recognized as etiologic agents. Recently, rotavirus and enterotoxigenic E. coli have been recognized as important pathogens in pediatric diarrhea in the tropics (1-3). Still more recently, Yersinia enterocolitica (4) and Campylobacter (5) have been implicated in a large proportion of enteric disease. Little is known of the relative significance of those pathogens in Bangkok. Enteroviruses also may cause sporadic outbreaks of diarrheal in pediatric populations (6).

MATERIALS AND METHODS: One hundred children with diarrhea and 100 controls without gastrointestinal disease were studied. Stools and urine were collected from children in both groups. Clinical histories were obtained at the time of specimen collection.

Bacteria in the stool specimens were isolated and identified by standard techniques (7). Chemical biotyping of the Enterobacteriaceae was performed using the Analytab Enteric (API-20E) system (8, 9). Principal organisms of interest were: Salmonella sp., Shigella sp., Vibrio sp., Campylobacter sp., Y. enterocolitica and E. coli. The prevalence of other bacteria including Plesiomonas sp., Aeromonas hydrophilia and Shigelloides was also determined. Serological confirmation of Salmonella, Shigella and Vibrio species was made using commercial antisera and procedure, as was serotyping of E. coli. (DIFCO, Detroit, Mich.).

Enterotoxigenic E. coli heat-labile toxin (LT) identification was made in the Y1 adrenal cell assay (10, 11) and heat-stable toxin (ST) in the suckling mouse assay (12, 13).

Rotavirus detection was performed by the enzyme-linked immunosorbent assay of Ghose (14). Enteroviruses were isolated from stool specimens by standard tissue culture diagnostic techniques (15).

Urine specimens were screened for antibiotics.

Paired sera were collected from the patient group with diarrhea for future assessment of antibody rise to LT by microtiter adrenal tissue culture technique and to SA-11 simian virus by complement fixation and enzyme-linked immunosorbent assay procedures.

Table I

<u>Children with Diarrhea</u>		<u>Well Children</u>
<u>Age</u>		
Mean	1 11/12 years	1 9/12 years
Range	1/12 - 5 years	1/12 - 5 years
<u>Sex</u>		
Males	67	51
Females	33	49
<u>Duration of Diarrhea</u>		
Mean	44 hours	4% (4/89) had diarrhea in
Range	2 - 72 hours	preceding two weeks
<u>Number of Stools within 24 hours</u>		
< 5	41	-
< 10	46	
< 15	9	
Not Recorded	4	
<u>Vomiting</u>		
Yes	88	-
No	12	
<u>URI</u>		
Yes	16	-
No	84	
<u>Rash</u>		
Yes	1	
No	99	
<u>IV hydration</u>		
Yes	98	
No	2	
<u>Character of Stool</u>		
Water	59	-
Mucous	36	
Blood	5	
<u>Temperature</u>		
< 38°C	60	-
38 - 39.9°C	35	
≥ 40°C	3	

Table 2
Bacterial Enteropathogens Isolated

<u>Isolate</u>	<u>Diarrhea</u>	<u>No Diarrhea</u>
<u>NAG Group I</u>	2	0
<u>V. cholerae</u>	2	0
<u>Salmonella</u> Group B	3	0
Group E	1	0
Group E ₂	1	0
Group E ₄	2	0
<u>Shigella</u>	9	1
dysenteriae	0	0
flexneri	7	0
sonnei	1	0
boydii	1	1
<u>Yersinia enterocolitica</u>	0	1
<u>Campylobacter</u>	4	2
<u>Aeromonas hydrophila</u>	11	10
<u>Plesiomonas shigelloides</u>	4	2
<u>EPEC</u>	18	21
02:K56	1	1
018a 018c:K77	3	3
020a 020b:K61	2	2
044:K74	0	1
055:K59	3	1
086a:K61	0	1
0111:K58	3	2
0112:K66	1	0
0113:K75	0	2
0124:K77	1	0
0125:K70	0	2
0126	2	4
0127:K63	1	1
0128:K67	1	1

Table 3 Enterotoxigenic E. coli

Children with Diarrhea

	LT-ST	LT	ST	Total
5A ^Δ	0	1	0	1
7A [○]	0	5	0	5
8A	0	7	0	7
14A	0	1	0	1
20A	3	4	2	9
30A	0	8	0	8
33A	0	10	0	10
34A	10	0	0	10
35A	0	4	0	4
45A	0	7	0	7
46A	3	6	0	9
57A	0	0	10	10
61A	0	5	0	5
62A ⁺	0	4	0	4
65A	0	2	0	2
66A	10	0	0	10
24A	10	0	0	10
76A*	0	4	0	4
78A	0	0	10	10
86A	0	0	10	10
91A	9	1	0	10
93A	1	0	0	1

Δ One E. coli 020a 020b:K61 isolated non-toxigenic

○ Five E. coli 0128:K67 isolated non-toxigenic

+ Two E. coli 0111:K58 isolated non-toxigenic

* Two isolates included in EPEC serotypes 018a 018c:K77

Controls

	LT-ST	LT	ST	Total
7C	1	0	0	1
14C	8	0	0	8
26C	0	8	0	8
28C	0	0	7	7
29C	0	0	4	4
30C	0	8	0	8
40C	0	3	0	3
42C	0	7	0	7
52C	9	1	0	10
82C	0	2	0	2
94A	10	0	0	10
95A	0	1	0	1

None of toxigenic isolates from controls of serotypes included in EPEC pools.

Figure 1

RESULTS:

1. The characteristics of the children with diarrhea and well controls are listed in Table I.
2. Bacterial enteropathogens isolated are listed in Table 2.
3. Enterotoxigenic E. coli isolates are listed in Table 3. Enterotoxigenic E. coli were isolated from 22/100 (22%) of children with diarrhea and 10/100 (10%) of well children.
4. Rotavirus was detected by ELISA technique in 27/92 (29%) of children with diarrhea and 2/96 (2%) of well children*.
5. Twenty-nine percent (29/100) children with diarrhea and fifty-three percent (22/41) of children without diarrhea had antibiotics detectable in their urine. There was no correlation between the history of taking or not taking antibiotics and the presence of antimicrobial agents in the urine.
6. Enteroviruses were isolated from 8/33 (24%) of children with diarrhea and 3/19 (16%) of controls. (Figure 1)

2. The Effect of Antibiotic Usage on the Prevalence of Bacterial Enteric Pathogens

OBJECTIVE: To determine if there is a relationship between consumption of antibiotics, prevalence of diarrhea, and infection with multi-resistant toxigenic E. coli.

BACKGROUND: Previous studies have shown that many enterotoxigenic E. coli in the Far East are resistant to multiple antibiotics (16). Furthermore, the plasmid(s) coding for enterotoxin can occasionally hybridize with plasmids coding for antibiotic resistance (17). To determine how frequently this occurs in nature, the rectal flora of a population known to be frequent consumers of antibiotic was compared to that of a control population.

METHODS: In May 1979, 1090 prostitutes in Angeles City, Republic of the Philippines, were interviewed and urine and rectal cultures were collected. Six hundred and one food handlers were also interviewed and urine and stool specimens were collected.

* 2/96 were +; 6/96 were + and need to be clocked (8/96) = 8%

Specimens were transported to Bangkok in Cary-Blair transport media. These specimens are in the process of being screened for enteric pathogens including tox⁺ E. coli. Twenty percent of the intestinal flora of each group is being tested for antibiotic resistance.

RESULTS: 28.6 percent of the prostitute population vs 2.2 percent of the food handlers have a history of taking antibiotics within the week prior to culture. Ten percent of the prostitute population vs < 1 percent of the food handlers had antibiotics detectable in their urine. 7.4 percent of the prostitutes admitted to having had diarrhea in the preceding week vs 1.8 percent of the food handlers. The bacteriological results are still not complete.

3. Etiology of Travelers' Diarrhea in Peace Corps Volunteers During Their First Five Weeks in Thailand

OBJECTIVE: To determine the etiology of diarrheal disease in Peace Corps volunteers (PCVs) within their first five weeks in Thailand.

BACKGROUND: Previous studies of travelers' diarrhea in tropical countries have demonstrated that enterotoxigenic (tox⁺) E. coli are the predominant pathogens (18,19,20). The antibiotic sensitivity patterns of this enteric pathogen has led some investigators to suggest the use of doxycycline as a prophylactic agent (21). One report, however, suggests that tox⁺ E. coli in the Far East may, in fact, be frequently resistant to multiple antibiotics and that resistance is R-factor mediated (22). To further determine the etiology of travelers' diarrhea in Thailand, PCVs were studied during their first five weeks in country. Evidence of infection with the following enteric pathogens is being sought:

1. Salmonella, Shigella, Vibrio
2. Plesiomonas shigelloides
3. Yersinia enterocolitica
4. Campylobacter
5. Tox⁺ E. coli
6. Ova and parasites
7. Rotavirus
8. Norwalk agent

The extent of resistance of the imported and acquired fecal flora is being determined.

METHODS: Thirty-five PCVs were interviewed within four hours of arrival in Thailand. Serum and stool specimens were collected within the next 24 hours (four specimens were cultured in Vermont and kindly examined by Dr. Samuel Formal, WRAIR). Each PCV was interviewed daily for the next five weeks, while they remained in U'thong, and 1-3 stool specimens were cultured from each individual during his or her episode(s) of diarrhea. Stools were streaked immediately on to selective media, frozen in dry ice, and emulsified in 10 percent formalin and PVA. After five and ten weeks in Thailand, additional stool specimens were collected.

RESULTS: Eighty-three percent (29/35) of the PCV group experienced diarrhea during the study period. The mean number of episodes (an episode as defined as diarrhea separated by two asymptomatic days) was 2.4, median 2.5, range 0-5. Assays are currently in progress. Diarrhea lasted for a mean of 2.6, median 2.8, range 1-12 days. One patient required hospitalization while two others were confined to their rooms for greater than 48 hours.

The general plan is to determine the etiology of diarrhea in this PCV group "Thai 66" and the extent of resistance of their enteropathogens. Further attempts to prevent diarrhea in the next PCV group "Thai 67" due to arrive in March 1980 will be determined after the data are completed. Various modes of prophylaxis, antibiotics, pectin, chlpromazine, will be evaluated in future PCV groups.

4. Etiology of Diarrhea in a Neonatal Nursery: Role of Maternal Stool as a Source of Infection for Their Infants

OBJECTIVES:

1. To correlate maternal with infant rectal flora within three days after delivery.
2. To determine the prevalence and etiology of diarrheal disease in the Phra Mongkutklao Hospital nursery.

BACKGROUND: Previous studies of the Phra Mongkutklao Hospital have described a high incidence of enteric pathogens in mothers at the time of delivery (23, 24). There has been a suggestion that mothers may be a source of enteric pathogens for their infants. The majority of these enteropathogens were enteropathogenic E. coli. Serotyping of these E. coli was not done

adequately nor were isolates tested for toxin production. Therefore, a pilot study (2 months) to include serotyping and tests for toxin production was undertaken.

METHODS: From April 26 until July 6, 1979, stool specimens were collected from 94 mothers and 84 infants (75 infant mother pairs). Stool specimens (1-3/infants) were also collected from 18 infants with diarrhea, in half of whom we were able to collect matching maternal specimens. Sixty stools were collected from 34 nursing personnel (26 twice) to determine if they were a source of enteropathogens.

Specimens were screened for Salmonella, Shigella, and enteropathogenic E. coli (EPEC) (by heating and retesting agglutinating isolates in the EPEC pools). Ten lac (+) colonies were picked from each culture and were tested for heat-labile toxin (LT) and heat-stable toxin (ST) in the Y-1 adrenal and suckling mouse assays (25, 26).

RESULTS:

1. The incidence of diarrhea was 4.6 percent (18/390) among the infants in the nursery during the study period.
2. Only one of 74 infant mother pairs shared the same enteropathogen (EPEC 0127a:K63).
3. Rotavirus was found in 1/18 infants with diarrhea but not in the mothers' stools.
4. Tox E. coli was isolated from 22 percent (4/18) of infants with diarrhea, but was not present in the mothers' stools.
5. Shigella were isolated from three nurses - nine percent (3/34).
6. Campylobacter (1/18) and EPEC (4/18) were isolated from infants with diarrheal stools. EPEC were isolated from 3/67 infants and 32/94 mothers without diarrhea. There was no statistical difference between the isolation rate of EPEC from infants with diarrhea and well controls. Campylobacter were not found in asymptomatic individuals. Yersinia enterocolitica were not found in this study population.

Data on Enteric Infection in a Neonatal Nursery, Army Hospital

Etiology of Neonatal Diarrhea

Total infants studied 84 cases.

Infants with diarrhea	18 cases (21.5%)
no diarrhea	66 cases (78.5%)

Causes of Diarrhea

<u>Campylobacter jejuni</u>	1	
<u>Enterotoxigenic E. coli</u> (ST+)	1	(10B-D 1)
(LT+)	3	(24B-D 2, 3, 81 B-D 1, 99 B-D 2)

Among 18 diarrheal infants, nine (50 percent) mothers' stools were examined while another nine cases (50 percent) were not cultured (because the infants developed symptoms after mothers were discharged.)

Nine pairs of mothers and diarrheal infants.

1. 7M EPEC 0125:K70; 0127:K63	7B, 7B-D1, 7B-D2, 7B-D3
<u>V. parahaemolyticus</u>	
<u>Pl. shigelloides</u>	
2. 10M EPEC 0127a:K63	10B EPEC 0128:K67
	10B-D1 <u>Enterotoxigenic</u>
	<u>E.coli</u> (ST+)
3. 23M	23B-D1
4. 34M EPEC 020a 020b:K84	34B-D1, B-D2, B-D3
<u>A. hydrophila</u>	
5. 44M EPEC 020a 020c:K61	44B, B-D1)
<u>A. hydrophila</u>	B-D2)- <u>Campylobacter</u>
	B-D3)
6. 45M	45B, B-D1, B-D2, B-D3
7. 60M <u>Pl. shigelloides</u>	60B-D1, B-D2
8. 95M	95B-D1, B-D2, B-D3
9. 99M	99B-D1
	B-D2 <u>Enterotoxigenic E.coli</u>
	B-D3 (LT+)

Nine diarrheal infants, no mothers' specimens

1. 24 B-D1
B-D2 Enterotoxigenic E. coli (LT+)
B-D3 E. coli (LT+)
2. 37 B-D1
B-D2
B-D3
3. 50 B-D1
B-D2
B-D3
4. 65 B-D1
B-D2
B-D3
5. 66 B-D1
B-D2
B-D3
6. 71 B-D1 020A 020c:K61
B-D2 020a 020c:K61
B-D3
7. 73 B-D1 020a 020c:K61
B-D2 020a 020c:K61
B-D3
8. 77 B-D1 020a 020c:K61
B-D2 020a 020c:K61
B-D3 020a 020c:K61
9. 81 B-D1 Enterotoxigenic E. coli (LT+)
B-D2
B-D3

Mothers who were positive with enteropathogens

- | | |
|-------------------------------------|--|
| 1. 7M <u>V. parahaemolyticus</u> | 7B-D neg. for <u>V. parahaemolyticus</u> |
| 2. 30MSh. <u>flexneri</u> 4 | 30B no diarrhea |
| 3. 48M <u>S. bialfra</u> (group E2) | 48B no diarrhea |

REMARKS Only 1 pair (9M-9B) EPEC 0128:K67 was isolated from both mother and infant (no diarrhea)

5. Resistance to Penicillin and Identification of Penicillinase Producing Neisseria gonorrhoeae from Clinical Isolates in Thailand

OBJECTIVES:

1. To monitor selected clinic populations reporting symptoms of gonococcal disease and having infections confirmed by laboratory culture.
2. To identify those isolates with resistance to penicillin (RSP).
3. To perform determinations of penicillin minimum inhibitory concentrations (MIC) from RSP isolates.
4. To identify the penicillinase-producing Neisseria gonorrhoeae (PPNG) and correlate with MIC findings.

BACKGROUND: Penicillin has historically proven an effective and low cost therapy for the treatment of uncomplicated gonococcal infections. Many new antibiotics are also active against gonococcal infections. Beta-lactamase positive strains of Neisserai gonorrhoeae have in general been shown to demonstrate in-vitro resistance to penicillin G, ampicillin, amoxicillin and carbencillin. Strains of gonococci isolated from East Asia have shown varied susceptibility to methicillin, oxacillin, nafcillin, cloxacillin and dicloxacillin whether the strain was identified as a penicillinase-producing or not. However, the over-all trend is towards constant or increasing antibiotic resistance (27). The surveillance of penicillin susceptibility and beta-lactamase activity of isolates does provide one guide for therapeutic recommendations and contributes information to the apparent trend of resistance.

From 1972, an increasing resistance to penicillin by N. gonorrhoeae has been demonstrated in Bangkok (28). Penicillinase-producing Neisseria gonorrhoeae (PPNG) identifications has been reported from 27 countries in Europe, Asia, Africa, Oceania, and North America to the World Health Organization (29). Epidemiological evidence suggests two separate focal origins of PPNG strains, the Far East and parts of West Africa (30). The potential of plasmid-initiated resistance as a factor with antibiotics other than penicillin, the risk of such plasmids entering organisms other than the gonococci and the spread of resistant strains to geographic areas of low incidence has made penicillin sensitivity

and PPNG identification a world-wide public health objective (31, 32).

METHODS: A total of 230 male and 175 female cases were studied, beginning in April 1978. All patients attended the Phra Mongkutklao Royal Thai Army Hospital venereal disease clinic or the Ban Chiwi clinic of the Bangrak (Public Health) Hospital for venereal disease examination. Patients were selected as demonstrating clinical symptoms of Neisseria gonorrhoeae infection or recent history of poor response to therapy (33). Male patient specimens were taken as urethral exudate and female specimens as cotton bud swabs from the cervical area. Specimens were immediately prepared for a microscopic gram stain examination and streaked on to Thayer-Martin agar (34) plates which were incubated for up to 72 hours at 37°C under 10 percent carbon dioxide. Gonococci were identified by gram stain, colonial morphology, oxidase reaction, and sugar fermentation (35).

Culture confirmed isolates were studied for the production of penicillinase using a penicillin disc diffusion technique, isolates being streaked on a cultured lawn of a penicillin susceptible Staph. aureus strain (27), and by the chromogenic cephalosporin test (36). Positive agreement between the techniques was required for PPNG identification.

Minimum inhibitory concentrations to penicillin were determined by plate dilution (28) using standardized culture suspensions and Thayer-Martin plate serial dilutions of penicillin G in concentrations of 0.06 through 24 micrograms per milliliter. Isolates with an MIC greater than 24 µg/ml were reported as such and those with less than 0.6 were considered susceptible.

RESULTS: The pattern of penicillin G MIC activity for the 405 isolates (Table 4; Figure 2) shows the trend of a simple gaussian-like distribution. Quarter year data (Table 5; Figure 3) did not show any notable variation over the year. The mean MIC and one standard error (SE), excluding penicillinase-producing isolates, for males was 0.799 ± 0.039 ; females 0.812 ± 0.032 and overall 0.805 ± 0.022 . Seventy-five isolates 52 from males and 23 from females, were identified as PPNG. All isolates with an MIC greater than or equal to 6 µg/ml were identified as penicillinase-producing (Table 4). The percentage of PPNG isolates was 22.6 percent of male isolates, 13 percent of female and 18.5 percent of the total 405.

Treatment regimens used in Bangkok on suspected venereal disease patients include spectinomycin (2-4 gm), ampicillin and

Table 4. Pattern of Penicillin G MIC ($\mu\text{gm/ml}$) Activity

MIC (Pen.G $\mu\text{gm/ml}$)	Male (n = 230) Cases	Female (n = 175) Cases	β -lactamase
0.06	0	0	-
0.12	3	3	-
0.24	13	7	-
0.36	18	15	-
0.48	23	19	-
0.60	26	19	-
0.72	17	17	-
0.84	17	14	-
0.96	12	16	-
1.08	21	10	-
1.2	9	15	-
1.5	9	13	-
1.8	4	2	-
2.1	3	2	-
2.4	2	0	-
3.0	1	0	-
6.0	1	3	+
12.0	0	0	
24.0	1	2	+
>24.0	50	18	+
TOTAL	230	175	
	=====	=====	

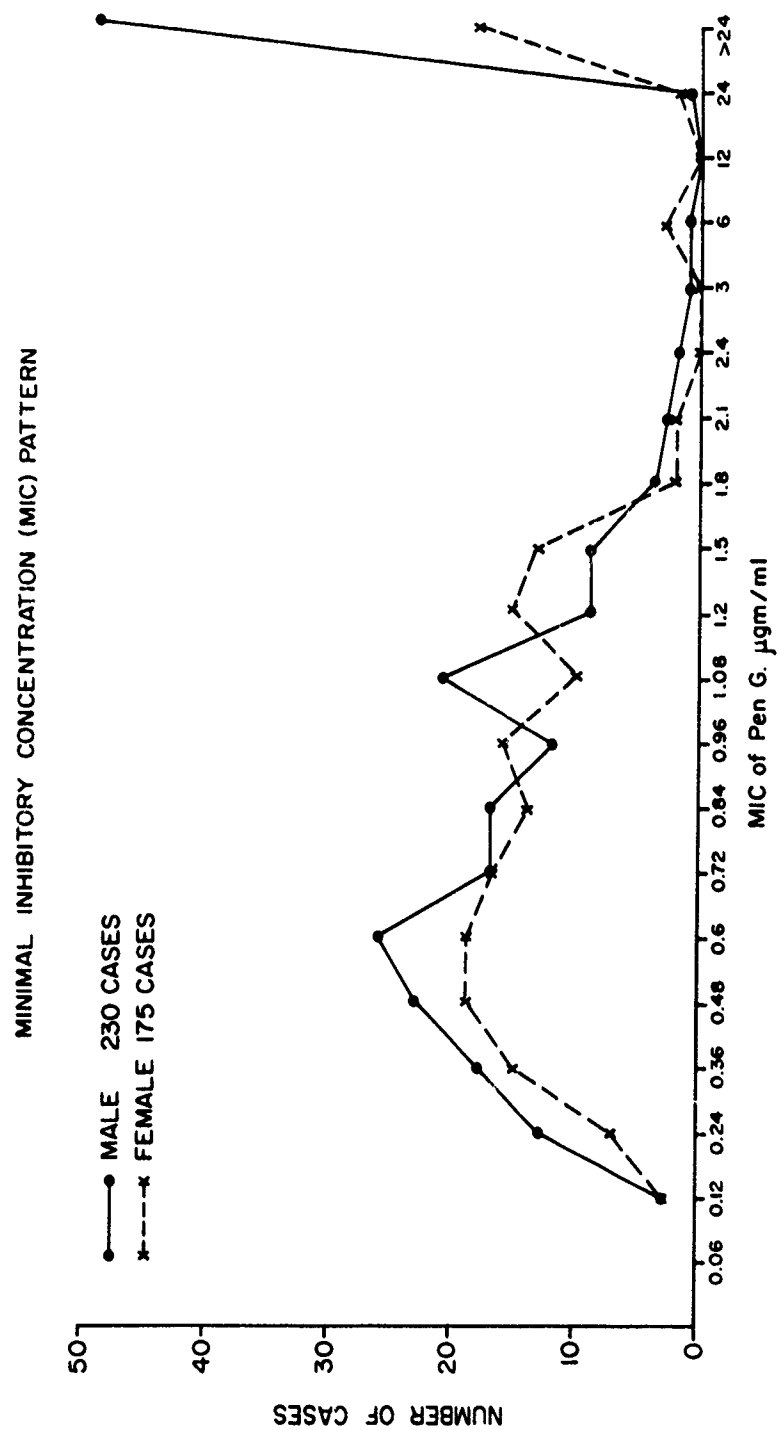
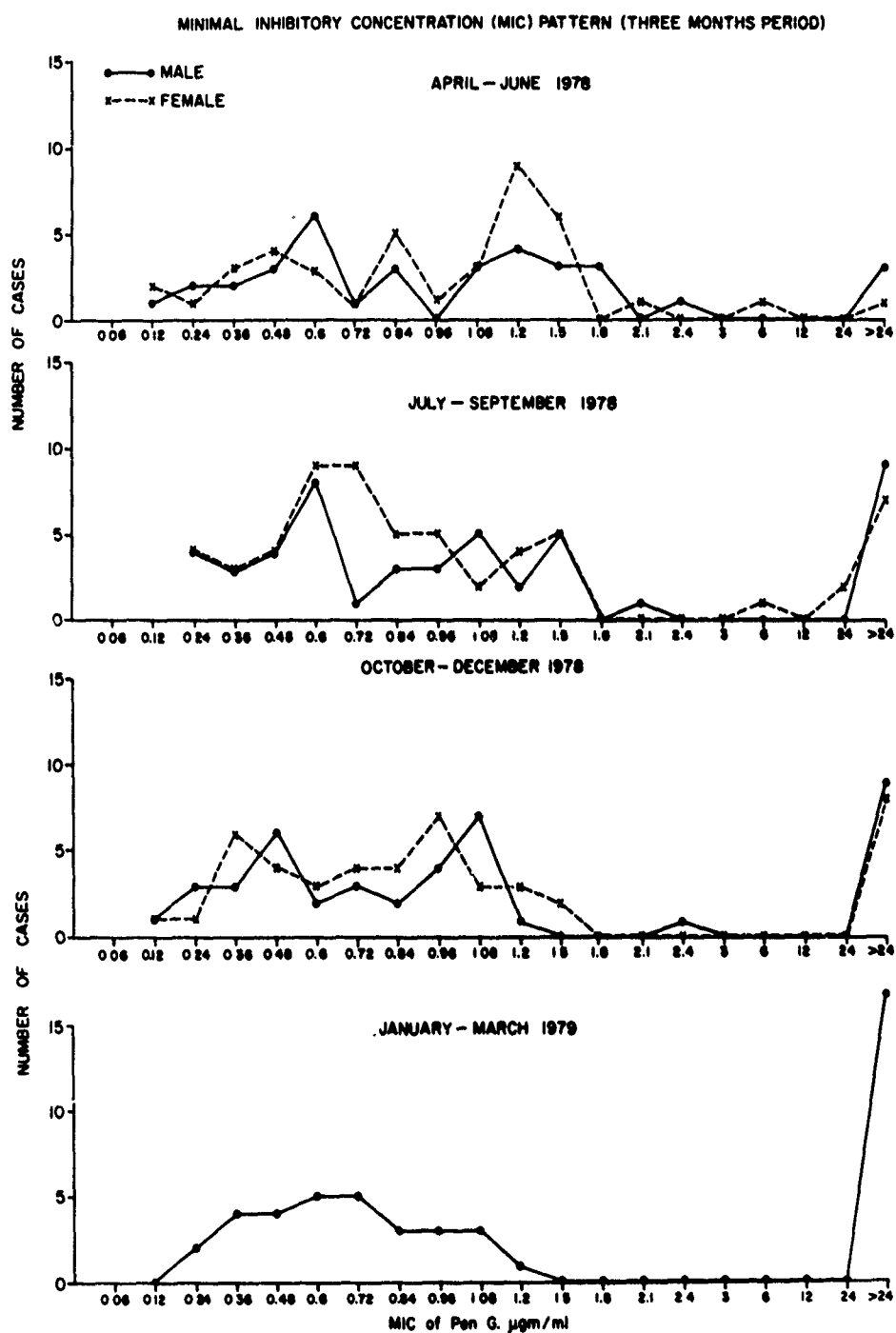


Figure 2

Table 5: MIC BY MONTHS

MIC µgm/ml	Mar 78		Apr-Jun 78		Jul-Sep 78		Oct-Dec 78		Jan-Mar 79		Apr-Jun 79		July 79	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F
0.12			1	2			1	1			1			
0.24	1		2	1	4	4	3	1	2	1	1			
0.36			2	3	3	3	3	6	4		4		1	
0.48		2	3	4	4	4	6	4	4	5	6			
0.6		5	6	3	8	9	2	3	5	2	5		1	
0.72	4	2	1	1	1	9	3	4	5		3			
0.84	2		3	5	3	5	2	4	3		3		1	
0.96	1	3		1	3	5	4	7	3		1			
1.08	1	2	3	3	5	2	7	3	3		1			
1.2			4	9	2	4	1	3	1		1		1	
1.5			3	6	5	5		2						
1.8	2	2	3											
2.1	2	1		1	1									
2.4			1				1							
3.0											1			
6.0		1	1	1		1								
12.0														
24.0						2					1			
>24	2	1	3	1	6	7	9	8	18	1	11		1	
TOTAL	15	19	36	41	45	60	42	46	48	9	39		5	
=====														



probenecid (3.5 gm), procaine-penicillin G and probenecid (4.8-6 million U, IM), or kanamycin (2 gm). Selection of the treatment regimen was based on clinical judgement, response and drug availability. The ampicillin or the procaine penicillin G and probenecid regimens were the most commonly prescribed. Studies in the United States have shown that PPNG strains have higher MICs for penicillin, ampicillin, erythromycin, tetracycline, and spectinomycin (37). The mean penicillin MIC in $\mu\text{g/ml}$, reported in Thailand during 1972, 1973, and 1974 (28) was 0.348, 0.432 and 0.63 respectively. Our two previous studies of selected clinical populations, excluding PPNG isolates (23, 39) exhibited a mean MIC and SE of 0.688 ± 0.044 and 0.883 ± 0.037 . In our present study, the mean penicillin MIC and SE was 0.805 ± 0.022 . During 1972-74, data were calculated deleting all MIC values above 1.2 $\mu\text{g/ml}$. If this exclusion were applied to our 1979 data, it would represent over 25 percent of the values used for calculation purposes. We reported during 1977 that 8 percent of our 105 study isolates were beta-lactamase positive and during 1978 that nine percent of 182 isolates were beta-lactamase positive. Of the present 405 cases, 75 (18.5 percent) were PPNG positive and had a MIC indicating penicillin resistance.

6. Epidemiological and Ecological Studies of Scrub Typhus in Royal Thai Army Field Training Facilities

OBJECTIVES:

1. To prospectively determine the susceptibility and the exposure risk of Royal Thai Army personnel to Rickettsia tsutsugamushi during field training exercises.
2. To determine the prevalence of Rickettsia tsutsugamushi in selected species of small mammals and chiggers from areas and habitats utilized by troops during training.
3. To determine if there is a seasonal effect influencing the susceptibility risk of Royal Thai Army personnel.
4. To evaluate the use of regional, habitat and seasonal data for predicting human exposure and risk potential to scrub typhus in Thailand.

BACKGROUND: In a previous report (23), the rationale for the establishment of a pilot project at Pak Chong, Nakhon Ratchasima Province, was presented. Preliminary results were presented in

the report cited; the present report involves data accrued during this reporting period.

METHODS: Sixty-five soldiers undergoing Special Forces training at Pak Chong, Nakhon Ratchasima Province, contributed blood samples before and after their training. Training consisted of four weeks of classroom and field problems and four days of jungle bivouac approximately 15-20 km. from the base camp. The troop training includes daily contact with forested or grassy areas and a bivouac in similar areas. Total field exposure was calculated for each individual and background information on age, rank, occupation (both in and outside of the military), home, travel and previous medical history was taken. The latter of the two blood samples was collected two weeks after the end of training. The blood samples were tested by the Weil-Felix (40) and by the indirect immunofluorescence tests (41). Sulfonamide level determination was performed on 15 samples to determine the level of Fansidar usage as a possible confounding variable.

Rodents and other small mammals were live-trapped in each habitat associated with troop training, and often in the actual training areas. Traps were baited with bananas and normally placed in 2-3 habitats each night. All small mammals collected were identified to species, sexed, aged, bled via cardiac or retro-orbital puncture and examined for ectoparasites. Rodent rickettsial serology will be done at AFRIMS by the IFA technique (41). Attached chiggers were gently scraped off and placed in vials containing 70 percent ETOH. Chiggers were counted and collated according to host species and habitat. Unengorged chiggers were collected in the troop training areas by black plates. Chiggers collected from black plates were kept alive in vials of water and sent to USAMRU-Kuala Lumpur, for rickettsia isolation by the micro direct fluorescent antibody (MDFA) technique (42).

RESULTS: All work this period was conducted in the base camp area at Ban Nong Tha Ku. This area is government owned, hilly and primarily covered with secondary evergreen forests, although more ecologically transient vegetation such as grass and early regenerating evergreen is also common. The basic soil type in the area is red-orange clay and the forest floors are fairly well preserved with considerable humus and leaf litter, indicating that fires are uncommon. Three basic habitats were identified in the area, i.e., tall grass, early regenerating evergreen and secondary evergreen. Because of troop field training exercises, trails were common in many areas of all 3 basic habitats, facilitating small mammal trapping.

Considerable precipitation fell in the study area during the period 24 Oct-17 Dec 1978, as compared to the previous report period (23), particularly the first trapping period in June 1978.

During the last period of work in camp (Dec 1978) temperatures dropped considerably, with early morning lows on several days in the range of 8-12°C, and daytime temperatures in the range of 18-23°C. Warmer temperatures also occurred during that period.

Entomological-rodent trapping studies occurred during the period 24 Oct-17 Dec 1978, and were conducted during 2 separate 10 day periods. The first period, 24 Oct-2 Nov, corresponded with the first portion of training for the test soldiers, while the second period, 8-17 Dec, corresponded with the latter part of training for the test soldiers. The test soldiers were in frequent contact with the 3 basic habitats during both periods of this study. Although it was not possible to calculate the ratio of their exposure to each basic habitat, more training occurred in the grass and secondary evergreen habitats than in the early regenerating evergreen.

A total of 486 small mammals of 15 species were collected from 2,364 trap-days (traps x 24 hr periods) during this study period (Table 6). The 3 basic habitats were not trapped equally (index trap-days): 53 percent in secondary evergreen, 37 percent in tall grass and 10 percent in early regenerating evergreen. Accordingly, more animals were caught in secondary evergreen than in the tall grass and early regenerating evergreen respectively. Furthermore, trapping efficiency was higher in the secondary evergreen (one animal per 4.3 trap-days) than in the tall grass (one animal per 5.5 trap-days) and the early regenerating evergreen (one animal per 6.5 trap-days).

Seven of the 15 species accounted for 96 percent (465/486) of the total number of small mammals captured: Rattus surifer, R. rattus, Tupaia glis, R. sabanus, R. bukit bukit, Menetes berdmorei and R. doratensia, in descending order of abundance, respectively. Most of these 7 species are commonly associated with a large number of chigger species in Thailand (43), including the most common vector of R. tsutsugamushi, L. (Lept.) deliense. Very obvious differences were detected in the numbers of species and individuals of given mammal species captured in the three habitats. While only 32.5 percent (158/486) of the total animals were captured in the tall grass, 93 percent (14/15) of the total

Table 6 Small mammals collected by habitat, in the Pak Chong Special Forces Training Camp (Ban Nong Tha Ku). 24 October - 17 December 1978.

Habitat	Trap days ¹	Mammals														Totals	
		<u>Bandicota savillei</u>	<u>Herpestes javanicus</u>	<u>Hylomys suillus</u>	<u>Menetes berdmorei</u>	<u>Mus cervicolor</u>	<u>Mus</u> sp.	<u>Rattus berdmorei</u>	<u>R. bukit bukit</u>	<u>R. exulans</u>	<u>R. koratensis</u>	<u>R. losea</u>	<u>R. rattus</u>	<u>R. sabanus</u>	<u>R. surifer</u>		<u>Tupaia glis</u>
Tall Grass	866	3	1	2	5	1	1	-	12	1	1	2	74	13	28	14	158
Early Regenerating Evergreen	246	-	-	3	3	-	-	-	2	-	-	-	6	4	12	8	38
Secondary Evergreen	1252	-	-	5	21	-	-	1	23	1	21	-	7	31	124	56	290
TOTALS	2364	3	1	10	29	1	1	1	37	2	22	2	87	48	164	78	486

¹ 24 hr period from 1200 hrs to 1200 hrs.

species captured came from this habitat. Nearly 60 percent (290/486) of the total animals were captured in the secondary evergreen, but only 67 percent (10/15) of the species were captured there, while nearly 47 percent (7/15) of the species were included in the 7.8 percent (38/486) of the total animals captured in the early regenerating evergreen.

Rattus rattus, the species usually considered most closely associated with L. (Lept.) deliense, was most common (85 percent 74/87) in the tall grass habitat. An additional five species, Bandicota savilei, Herpestes javanicus, Mus cervicolor, Mus sp. and Rattus losea, were captured only in the grass. On the other hand, 95 percent (21/22) of Rattus koratensis, 76 percent (124/164) of R. surifer, 72 percent (56/78) of Tupaia glis, 72 percent (21/29) of Menetes berdmorei and 65 percent (31/48) of R. sabanus were collected in the secondary evergreen habitat. Only the insectivore-predator, Hylomys suillus, seemed to be fairly equally distributed in the three different habitats, although more specimens were collected in the early regenerating evergreen than would be expected.

A total of 148 unengorged chiggers were collected from 19 black plate (BP) collections during this period, of which 109 were screened and 8.3 percent (9) were found positive for Rickettsia tsutsugamushi (Table 7.) by the MDFA technique (42). The infected specimens included 14.3 percent (6/42) of the L. (Lept.) deliense screened and 5.8 percent (3/52) of the Odontacarus sp. screened. The last species is apparently new to science. Actually, six of the 19 BP collections were made during the period 24 Oct-2 Nov, and the remaining 13 BP collections were made during the period, 8-17 December. Chigger collection, habitat and rickettsial isolation data for these two periods are summarized in Tables 8 and 9. These data show that chiggers infected with R. tsutsugamushi were collected during both periods and in two habitats, i.e., early regenerating evergreen (last period) and secondary evergreen (both periods). Furthermore, the chigger species composition (based only on BP collections) was completely different for the two periods. Only L. (Lept.) miculum arvinum and deliense were collected in the first period, while only Helenicula sp., L. (Lept.) Sp.D, L. (Trom.) paniculatum and Odontacarus sp. were collected during the last period.

Collections of engorged chiggers were made from all parasitized animals. These specimens have been slide mounted and are currently being identified. The rodent sera collected during

Table 7. Chiggers collected and Rickettsia tsutsugamushi isolations from the Pak Chong Special Forces Training Camp, 24 Oct. - 17 Dec. 78.

Chigger Species	No. of Chiggers			
	Coll.	Screened	Chigger Infected	
			No.	Percent
<u>Helenicula</u> sp.	6	6	0	0
<u>L. (L.) deliense</u>	52	42	6	14.3
<u>L. (L.) miculum arvinum</u>	3	3	0	0
<u>L. (L.) sp.D.</u>	1	1	0	0
<u>L. (T.) paniculatum</u>	5	5	0	0
<u>Odontacarus</u> sp.	81	52	3	5.8
	148	109	9	8.3

Table 8 Black plate chigger collections, habitat and rickettsial isolation data from the Pak Chong Special Forces Training Camp, 24 Oct. - 2 Nov. 1978.

Species	Secondary Evergreen (5 coll.)			Early Regenerating Evergreen (1 coll.)		
	Chiggers			Chiggers		
	No.	Screened	Infected % (No.)	No.	Screened	Infected % (No.)
<u>L. (L.) deliense</u>	51	41	14.6(6)	1	1	0
<u>L. (L.) miculum arvinum</u>	3	3	0	-	-	-
TOTAL	54	44	13.6(6)	1	1	0

Table 9 Black plate chigger collections, habitat and rickettsial isolation data from the Pak Chong Special Forces Training Camp, 8-17 Dec. 1978.

Species	Secondary Evergreen (9 coll.)			Early Regenerating Evergreen (4 coll.)		
	Chiggers			Chiggers		
	No.	Screened	Infected % (No.)	No.	Screened	Infected % (No.)
<u>Helenicula</u> sp.	5	5	0	1	1	0
<u>L. (L.)</u> sp.D.	-	-	-	1	1	0
<u>L. (Trom.) paniculatum</u>	5	5	0	-	-	-
<u>Odontacarus</u> sp.	54	37	5.4(2)	27	15	6.7(1)
TOTAL	64	47	4.3(2)	29	17	5.9(1)

this period and the previous annual progress report period are currently being examined for R. tsutsugamushi antibody levels.

Blood specimens on 65 soldiers were tested prior to the initiation of training and three (4.6 percent) were seropositive by the IFA technique. Fifty-nine of these soldiers (including the three with pre-existing antibody) were subsequently re-examined after training and none were positive at that time.

It is apparent from the lack of antibody against R. tsutsugamushi in this population (less than 5 percent) that the soldiers tested have not been exposed to scrub typhus in the recent past. The full duration of antibody retention is not known, but experimental infections in the silver-leaf monkey model have shown evidence of a low level of antibody up to two years following an infection (44). The loss of antibody in the three soldiers who had evidence of antibody prior to the initiation of training in a blood sample taken six weeks later attests to the transient nature of this antibody. The absence of infections in this human population, in spite of the close proximity to infected chiggers, indicates insufficient contact between the humans and the chiggers for infections to occur. Consequently, this area provides a poor site for the continuation of this project beyond the pilot stage.

Unfinished aspects of this project will be completed in the near future, at which time the studies in Pak Chong will be terminated.

7. Seroepidemiologic Survey of R. tsutsugamushi
Infection in a Rural Thai Village

OBJECTIVES:

1. To determine the prevalence of antibody to R. tsutsugamushi in a well-defined rural Thai population.
2. To determine strain types of R. tsutsugamushi present and infective in Thailand.

BACKGROUND: Ban Tablan, Prachinburi Province, Thailand, is a relatively isolated rural village approximately 250 km. northeast of Bangkok. The population is primarily engaged in subsistence agriculture, hunting and logging activities. The living quarters are elevated wood and thatch buildings with partially closed in sleeping areas. Deep evergreen forest penetration is confined to the adolescent and adult males during logging activities, but

fruit-gathering by the women and play activities by the children allow some thin forest penetration by all members of society. Open sandals and clothing allowing bare arms and legs are common among all villagers.

METHODS: Sera were collected in October-December 1976 in conjunction with a seroepidemiologic survey of the entire village. A 20 percent random sample, stratified by age, was selected.

An indirect immunofluorescence technique was used to assess all serum for antibody (45). Antigen, Karp, Kato and Gilliam strains, was obtained from the Department of Rickettsiology, USAMRU, Institute for Medical Research, Kuala Lumpur, Malaysia, as a 20 percent yolk sack membrane suspension prepared from specific pathogen-free hen's eggs. Serum was first screened at an initial 1:50 dilution against an antigen pool of the three strains. Positive screen specimens were subsequently titrated from 1:50 against the antigen pool and the individual antigens. The fluorescent conjugate used was a horse immunoglobulin anti-human immunoglobulin. Readings of the fluorescence were performed with a Leitz Orthoplan research microscope equipped with fluorescence technique objectives, either vertical or transmitted light, and at 40X dry power. Filters and light sources were combined to produce an ocular maximum fluorescence intensity close to 510 millimicrons.

RESULTS: The prevalence of antibody to R. tsutsugamushi in this Thai village is shown in Table 10. Overall, nearly two-thirds of villagers had evidence of antibody. For females, the prevalence of antibody did not change significantly between age groups. However, the prevalence of antibody in males 15 years of age and older was significantly different from the younger males (0.873 vs 0.371; $p < 0.001$). The prevalence of antibody in these younger males was also considerably less than that of their female contemporaries (0.690, $p < 0.02$).

Of the 147 positive specimens, 122 had two or more strain titers that were indistinguishable. Only 25 had either a monospecific positive titer (4) or a single titer level four or more times greater than any other positive titer (46) (21). Table 11 presents the distribution of serologic results over the three prototype strains of R. tsutsugamushi. Twelve of the type specific titers were in females, ages 1 to 53 years. The age range of the 13 males was exactly the same. The geometric mean titer (GMT) of

TABLE 10

Prevalence of Antibody to R. tsutsugamushi in Residents of
Ban Tablan, Thailand, by Indirect Immunofluorescence Test 1976

Age	MALE		FEMALE		TOTAL	
	No. of individuals tested	No. (%) of individuals positive	No. of individuals tested	No. (%) of individuals positive	No. of individuals tested	No. (%) of individuals positive
1-4	8	4(50)	8	6(75)	16	10(63)
5-9	17	6(35)	13	9(69)	30	15(50)
10-14	10	3(30)	8	5(63)	18	8(44)
15-19	10	9(90)	11	8(73)	21	17(81)
20-29	10	8(80)	18	13(72)	28	21(75)
30-39	11	10(91)	20	13(65)	31	23(74)
40-49	12	10(83)	15	10(67)	27	20(74)
50-59	12	11(92)	13	9(69)	25	20(80)
60+	8	7(88)	9	6(67)	17	13(76)

TABLE 11

Results of Indirect Immunofluorescence
Serology Ban Tablan, Thailand 1976

	Karp	Kato	Gilliam
Total positive titer	147	141	141
Total type specific titers	13	2	10
GMT of positive titers	1:263	1:189	1:188

the various age groups showed only negligible difference and demonstrated no consistent pattern or progression by age. There was no difference between the overall level of antibody titers in males and females, but females tended to have a slightly lower GMT than their male contemporaries.

A manuscript of this (completed) project has been submitted for clearance.

8. Ectoparasite and Rickettsia tsutsugamushi Studies in Thailand

OBJECTIVE: To establish and describe the chiggers and ticks that are vectors or potential vectors of human pathogens in Thailand, and determine the geographical distribution of Rickettsia tsutsugamushi in natural populations of chiggers in Thailand.

BACKGROUND: This is a continuation of previously reported studies (23). Earlier work during the period 1961-1972 served as the basis for a number of publications, in which Lakshana (47) and Lekagul and McNeely (48) have established a firm taxonomic base on which epidemiological-ecological studies on Rickettsia tsutsugamushi in Thailand can proceed. Recent emphasis has shifted to the distribution of strains of R. tsutsugamushi that occur in vector chiggers in Thailand. However, problems still exist in identifying chigger specimens collected in Thailand, hence taxonomic studies on chiggers are continuing.

METHODS: Ectoparasites are collected from live trapped rodents and other small mammals by removal with forceps, by scraping or by holding the animals alive over a pan of water and allowing engorged ectoparasites to drop into the water. Engorged chiggers are normally preserved in alcohol and mounted on slides for study. Chiggers used for R. tsutsugamushi isolation attempts are preferably unengorged. Unengorged chiggers are usually found in leaf litter, on rotten logs and other favorable habitats frequented by rodents and other small mammals, and are easily collected by using 5" x 5" formica black plates. Collected engorged chiggers are placed and kept alive in vials of water, which are then shipped to USAMRU-Kuala Lumpur for rickettsia studies. A technique using direct immunofluorescence has recently been developed to detect rickettsia in naturally infected mites (49). Using this technique, the internal contents of each unengorged chigger can be screened for nine different strains of R. tsutsugamushi, i.e. Karp, Gilliam, Kato TC 586, TA 678, TA 686, TA 716, TA 763 and TH 1817.

After the internal contents of the chigger has been tested for rickettsia, its exoskeleton is mounted in Hoyer's mounting media on a slide for identification.

RESULTS: Between 25 Oct and 21 Dec 1978, 1,379 unengorged chiggers of 12 species were collected in Thailand by the black plate method and sent to USAMRU-Kuala Lumpur for rickettsia detection and subsequent identification (Table 12). These specimens were collected in two provinces, Chiangmai and Nakhon Ratchasima (two areas-Pak Chong and Sakaerat). The five most commonly collected species were L. (L.) scutellare, L. (L.) deliense, L. (L.) miculum arvinum, Odontacarus sp. and L. (T.) paniculatum, in descending order of abundance. Four new species, Helenicula sp., L. (L.) sp. D, L. (L.) sp. G and Odontacarus were also identified during this period, and represented 7.6 percent of the total specimens collected. Of the 12 species collected, 828 specimens of 10 species were screened in Kuala Lumpur for Rickettsia tsutsugamushi, and six percent (50/828) were positive (Table 13). A total of six species were positive for R. tsutsugamushi from the two provinces, with L. (L.) deliense and scutellare positive in both provinces, while L. (L.) miculum arvinum was positive only in Chiangmai and E. wichmanni, L. (T.) paniculatum and Odontacarus sp. positive only in Nakhon Ratchasima Province. A total of 5.1 percent (20) of chiggers screened from Chiangmai were positive while 6.8 percent (30) of chiggers screened from Nakhon Ratchasima were positive.

The sources and habitats for the chiggers screened for R. tsutsugamushi from Thailand during this period are shown in Table 14. Initially it would appear that the primary foci for infected chiggers are primary and secondary evergreen forests. However, only a few black plate collections were made from the other three basic habitats (early regenerating evergreen, Pa Daeng (dry dipterocarp) forest and grass). Additional collections from all the habitats are needed before infection rates per habitat can be properly interpreted; however, four of the five habitats analyzed here contained infected chiggers. Probably most, if not all, of the basic habitats in Thailand contain chigger species harboring R. tsutsugamushi.

Several chigger species appear to be highly seasonal and found only during the cool season in Thailand. These species, Helenicula sp., L. (L.) scutellare, and Odontacarus sp., have been collected in Thailand only between the months of November to February. Of these, only Helenicula sp. was not found infected with R. tsutsugamushi. Leptotrombidium (L.) scutellare is a known

Table 12 Unengorged chiggers captured by black plate collections in Thailand between 25 Oct.-21 Dec. 1978 and sent to USAMRU- Kuala Lumpur for rickettsia isolation.

Chigger Species	Black Plate Collections Per Location	Chiang Mai (19)	Nakhon Ratchasima		TOTAL (63)
			Pak Chong (19)	Sakaerat (25)	
<u>Eutrombicula wichmanni</u>		-	-	23	23
<u>Helenicula</u> sp.		-	6	12	18
<u>Lept. (L.) deliense</u>		28	52	35	115
" " <u>fulleri</u>		1	-	-	1
" " <u>miculum arvinum</u>		77	3	2	82
" " <u>scanloni</u>		1	-	-	1
" " <u>scutellare</u>		394	-	591	985
" " <u>striatum</u>		-	-	1	1
" " sp. D ¹		-	1	1	2
" " sp G ¹		3	-	-	3
<u>L. (Trom.) paniculatum</u>		-	5	61	66
<u>Odontacarus</u> sp.		-	81	1	82
TOTALS		504	148	727	1,379

¹
New undescribed species

Table 13 Source and species of Thai chiggers that were screened for Rickettsia tsutsugamushi from collections during the period 25 Oct. - 21 Dec. 1978.

Location (Collection Dates) - Chigger Species	Specimens		
	Collected	Screened	Infected %(No.)
<u>Chiang Mai</u> (22 Nov.-4 Dec. 1978)	—	—	—
- <u>Lept. (L.) deliense</u>	28	24	8.3(2)
- " " <u>fulleri</u>	1	1	0
- " " <u>miculum arvinum</u>	77	56	1.8(1)
- " " <u>scanloni</u>	1	1	0
- " " <u>scutellare</u>	394	308	5.5(17)
SUBTOTALS	501	390	5.1(20)
<u>Nakhon Ratchasima</u> (25 Oct.-21 Dec.1978)	—	—	—
- <u>Eutrombicula wichmanni</u>	23	15	13.3(2)
- <u>Helenicula</u> sp.	18	9	0
- <u>Lept.(L.) deliense</u>	87	64	10.9(7)
- " " <u>miculum arvinum</u>	5	5	0
- " " <u>scutellare</u>	591	234	7.3(17)
- " " sp. D	2	1	0
- " (Trom.) <u>paniculatum</u>	66	58	1.7(1)
- <u>Odontacarus</u> sp.	82	52	5.8(3)
SUBTOTALS	874	438	6.8(30)
TOTALS	1,375	828	6.0(50)

Table 14 Source and habitats for Tnai chigger species screened for Rickettsia tsutsugamushi between 25 Oct. - 21 Dec. 1978.

Source - Species	Habitat - specimens		Primary Evergreen		Secondary Evergreen		Early Regenerat- ing Evergreen		Dry Dipterocarp Forest		Grass	
	No. Screened	Infected Z(No.)	No. Screened	Infected Z(No.)	No. Screened	Infected Z(No.)	No. Screened	Infected Z(No.)	No. Screened	Infected Z(No.)	No. Screened	Infected Z(No.)
<u>Chiang Mai (22 Nov. - 4 Dec. 78)</u>												
- <u>Lept. (L.) delense</u>	-	-	24	8.3(2)	-	-	-	-	-	-	-	-
- " " <u>fulvipes</u>	-	-	1	0	-	-	-	-	-	-	-	-
- " " <u>arvanum</u>	54	1.9(1)	2	0	-	-	-	-	-	-	-	-
- " " <u>scutellare</u>	-	-	1	0	-	-	-	-	-	-	-	-
- " " <u>scutellare</u>	153	5.9(9)	155	5.2(8)	-	-	-	-	-	-	-	-
<u>SUBTOTAL</u>		207	4.8(10)	183	5.5(10)	-	-	-	-	-	-	-
<u>Kachon Katchanawima</u>												
<u>(25 Oct. - 2 Dec. 78)</u>												
- <u>Eutrochium wachmanni</u>	-	-	-	-	-	-	-	-	-	-	15	13.3(2)
- <u>Heptacaulis sp.</u>	-	-	5	0	1	0	3	0	-	-	-	-
- <u>Lept. (L.) delense</u>	19	0	41	4.6(6)	1	0	-	-	-	-	3	33.3(1)
- " " <u>arvanum</u>	2	0	3	0	-	-	-	-	-	-	-	-
- " " <u>scutellare</u>	254	7.3(17)	-	-	-	-	-	-	-	-	-	-
- " " <u>sp.D</u>	-	-	-	-	1	0	-	-	-	-	-	-
- <u>Lept. (L.) paniculatum</u>	52	1.9(1)	5	0	-	-	1	0	-	-	-	-
- <u>Odontacarus sp.</u>	-	-	37	5.4(2)	15	6.7(1)	-	-	-	-	-	-
<u>SUBTOTAL</u>		307	5.9(18)	91	8.8(8)	18	5.6(1)	4	0	18	16.7(3)	
<u>TOTALS</u>		514	5.4(28)	274	6.6(18)	19	5.6(1)	4	0	18	16.7(3)	

Table 15 Summary of chigger species captured in black plate collections in Thailand and screened for Rickettsia tsutsugamushi during the period, June 1977 to December 1978.

No.	Species	Specimens		
		Collected	Screened	Infected %(No.)
1	<u>Asch. (L.) indica</u>	1	1	—
2	<u>Eutrombicula wichmanni</u> ¹	23	15	13.3(2)
3	<u>Gahrliepia (G.) mirabilis</u>	2	—	—
4	<u>Helenicula</u> sp. ²	18	9	—
5	<u>Lept. (L.) deliense</u>	2,018	929	9.4(87)
6	" " <u>fulleri</u>	22	1	—
7	" " <u>miculum arvinum</u> ¹	163	121	7.4(9)
8	" " <u>scanloni</u>	1	1	—
9	" " <u>scutellare</u> ¹	985	542	6.3(34)
10	" " <u>striatum</u>	1	—	—
11	" " sp. A ^{1, 2}	119	39	17.9(7)
12	" " sp. B ^{1, 2}	4	4	50.0(2)
13	" " sp. C ²	16	1	—
14	" " sp. D ²	4	2	—
15	" " sp. G ²	3	—	—
16	<u>Lept. (L.) paniculatum</u> ¹	77	59	1.7(1)
17	<u>Microtrombicula chamlongi</u> ¹	6	2	50.0(1)
18	<u>Odontacarus</u> sp. ^{1, 2}	82	52	5.8(3)
19	<u>Siseca rara</u>	16	—	—
20	<u>Walchiella oudemansi</u>	68	—	—
21	" <u>traubi</u>	1	—	—
TOTALS		3,630	1,778	8.2(146)

¹ species not previously recorded as infected with R. tsutsugamushi in Thailand

² new undescribed species

vector of R. tsutsugamushi in other areas of Southeast Asia, but previous to this study, has not been found infected in Thailand.

Table 15 summarizes the chigger species captured in black plate collections in Thailand and screened for R. tsutsugamushi since this project was initiated in June 1977. Included are chigger-rickettsial data from the Pak Chong study described elsewhere in this Annual Report. At that time only L. (L.) deliense had been incriminated as a vector of R. tsutsugamushi in Thailand. One and one half years later 8.2 percent (146) of 1,778 chiggers screened for R. tsutsugamushi infections have been found positive. Furthermore, nine of 15 species screened contained rickettsial infections, indicating a much larger number of potential vectors of this human pathogen in Thailand than previously suspected. The role of at least 7 of the nine infected species found here in the zoonotic maintenance of this pathogen is currently unknown. Species of the subgenus Leptrotrombidium are usually considered of primary importance in the transmission of this pathogen to man (50) and five of the infected species found in this study belong in this subgenus. However, infected species of three other genera, Eutrombicula, Microtrombicula and Odontacarus, and one species in the subgenus, Leptotrombidium (Trombiculindus), were also found. Generally, the species found infected with R. tsutsugamushi during this study fall into Nadchatram's (51) ecological group I, i.e., those species which affected man or have a better opportunity of coming in contact with him. According to the current interpretation of Nadchatram's groupings, only Lept. (T.) paniculatum and Microtrombicula chamlongi possibly would not fall into group I. However, these two species were not included in Nadchatram's study.

The large number of R. tsutsugamushi isolations made during this study were only possible because of the development of new techniques (49). Based on the attached data, the advantages of the MDFA technique in conjunction with the collection of unengorged chiggers on black plates are very obvious. Although the current project is scheduled for termination, these data suggest an obvious need for the continuation of similar studies.

A total of seven new species of chiggers were identified during this study, of which three were found infected with R. tsutsugamushi. Currently, the descriptions and illustrations for 12 new species of the genus Leptotrombidium (Lept.) have been completed (including one new species found in this study) and those for two additional species are being completed in preparation for

publication. The other six new species found in this study will be described and prepared for publication in the near future.

A checklist of the Ticks of Thailand, containing 54 species in 10 genera is nearing completion. Of those, 23 have not been reported previously from Thailand. A host list is also included.

The biological and taxonomic efforts of this study are continuing and additional rickettsial isolation studies are anticipated.

9. A Peroxidase Enzyme-Linked Paper Immunosorbent Technique (PELISA) Compared to Microimmunofluorescence (MIFA) for the Detection of Human Serum Antibodies to the Rickettsia, Rickettsia tsutsugamushi.

OBJECTIVES:

1. To design a technique which is technologically simple, inexpensive, demands little skill and presents potential for use in a relatively unsophisticated clinical setting.
2. To use the same antigens for the PELISA technique as for the MIFA procedure and compare the two.

BACKGROUND: Serodiagnosis of Rickettsia tsutsugamushi infection in clinical situations is usually accomplished by the Weil-Felix reaction detecting agglutinins to the OXK strain of Proteus mirabilis. The technique lacks specificity and sensitivity (52) and subsequent infections do not appear to demonstrate increased Weil-Felix titers (53). The complement fixation (CF) test does not give significant titers with acute-phase sera without endemic regional homologous strains of the rickettsia included as a part of the antigen (54,55).

Indirect immunofluorescence (IFA) procedures have become the accepted serodiagnostic technique because of their sensitivity in detecting group antigens as well as homologous strains of the scrub typhus rickettsia (56, 57). Adaptation of the IFA test to the microimmunofluorescent (MIFA) technique placed the fluorescent microscope technique in the position of being an acceptable tool for routine serodiagnosis and studies of the disease epidemiology (58, 59, 60). The Weil-Felix reaction, complement fixation test and microimmunofluorescent procedures are all complicated by the difficulty in preparing specific antigens (61, 62, 55). A microplate enzyme-linked immunosorbent technique has been used and is reported to offer an acceptable alternative to using the MIFA procedure (56).

In most clinical situations, the only technique available for laboratory diagnosis remains the Weil-Felix reaction, this is due to the expense of equipment and lack of skilled personnel to perform the other laboratory tests. During our studies of human serum antibody to the scrub typhus rickettsia, we decided to develop an enzyme-linked immunosorbent assay (ELISA) concurrent with our MIFA studies as an alternative immunochemical laboratory procedure.

METHODS: Antigen: Antigens for the Karp, Kato and Gilliam strains of R. tsutsugamushi, obtained from the Department of Rickettsiology, USAMRU, Institute for Medical Research, Kuala Lumpur, Malaysia, were prepared from specific pathogen free hen's eggs (SPAFAS, Inc., Norwich, Conn.) as a 20 percent yolk sac suspension in Synder's diluent (63). Aliquots of the preparation were stored at -70°C after determining the procedural working dilution. Working dilutions were determined, using the same diluent, which would yield approximately 1,000 organisms per 500X microscope field and titer by MIFA, to end point, with a final 1:50 dilution of high titer human sera. When testing was performed, the aliquot was thawed to 37°C , kept on wet ice, and prepared to the predetermined working dilution. The working preparations were used as equal volume pools and as single antigens for the MIFA method of Robinson (64) as well as for the ELISA technique. Negative serum controls, normal yolk sac suspension, and serum diluent were evaluated concurrently with the human sera.

Sera: Human serum from Thailand and Malaysia represented indigenous persons potentially exposed to scrub typhus; positive control sera were from patients infected with R. tsutsugamushi. Six groups of 98, 105, 10, 50, 58, and 30 sera were designated Tablan 1, 2, 3 (TP1, TP2, TP3), Pak Chong 2, 3 (PC2, PC3 and (KLX). All were stored at -20°C prior to testing. Serum tested by both procedures was initially diluted to 1:50 and screened against pooled antigen. Positive sera were then titered against the pool as well as against the separate antigens in 2-fold serum serial dilutions of 1:100, 1:200, 1:400, 1:800 and 1:1600. Control sera were defined as positive or negative at a 1:50 dilution. A test positive serum was read relative to the controls and positive titers as positive at 3 to 4 serial dilutions of the serum. For the MIFA procedure 28 sera and two controls were tested per microscope slide. Ten sera and two controls per slide were used for the ELISA technique.

MIFA procedure: Training for the performance of the test was obtained in Kuala Lumpur using the MIFA method of Robinson (64) and was performed in Bangkok with no deviation from the essential procedural steps. The method essentially prescribes appropriately diluted antigens and controls, placed as 30 pen nib dots on a microscope slide, fixed in acetone, incubated at 37°C with an initial 1:50 (serum: phosphate buffered saline) diluted specimen per antigen dot and again incubated with a fluorescence conjugated horse anti-human immunoglobulin. Phosphate buffered saline (PBS) was utilized for all intermittent washing steps. Microscopic fluorescence examination was performed with a Leitz Orthoplan research microscope, equipped with fluorescence technique objectives, at 40X dry power. Filter and light sources were combined to produce a maximum intensity ocular emission fluorescence at 525 millimicrons.

PELISA technique: Antigen was applied as a single one lambda drop on a microscope slide, fixed in chloroform: methanol, dried and sandwiched with a five lambda test serum drop (diluted 1:50), incubated, washed, dried and sandwiched with an anti-human IgG peroxidase conjugate (diluted 1:400), incubated, washed and dried before direct application of a substrate saturated filter paper. The enzyme reaction was visually monitored as production of a purple-brown product and terminated by removal of the filter paper holding to remaining substrate and product produced. This paper was quickly dried and retained as a permanent record of the test. All sera, as in MIFA, were first screened against pooled antigen. A positive serum was then titered against the pool and also titered against the individual antigens rendering separate titers for the pooled and individual antigens. Procedural details were as follows:

One lambda drops of working antigen, delivered by an Eppendorf automatic hand pipette, were spotted on slides precleaned with a mixture of chloroform N.F. (Allied Chemical, Morristown, N.J.): methanol ACS (Eastman Kodak, Rochester, N.Y.), 2:1 by volume (C:M) and air dried 1 hr. to approximately 5 mm diameter circular spots, 12 spots to a slide in 3 rows, 4 spots per row. After air drying, the spotted slides were fixed by immersion in C:M at 4°C for 10 min and again air dried. The antigen prepared slides were immediately used or stored in a desiccation container at -20°C and used within two weeks. Slides were general laboratory use 75 mm x 25 mm glass with one frosted end (Propper Mfg Co., Inc., L.I.C., N.Y.) Serum was diluted with a 1 percent Tween 20:0.5 M NaCl solution, pH adjusted to 7.4 with 0.1 M K_2HPO_4 (65). Five lambda of the diluted serum was spotted at

room temperature to cover the dry antigen spot on the microscope slide, maintaining an approximate 5 mm diameter circular antigen-serum surface area and incubated on a damp sponge humidified at 37°C for 30 minutes. The slide was then washed at room temperature with a pH 7.2-7.4 phosphate buffered saline (PBS) (66) three times, 5 minutes each time, by soaking in a histopathology Coplin jar which was emptied and refilled with fresh PBS between the successive soaks. Following this wash the slides were dried using an unheated hair dryer. Each slide was carefully cleaned around the antigen-serum spots with a dry cotton tipped applicator to remove any traces of residual serum which would interfere with the edge surface tension integrity of later sandwiched drop applications.

Anti-human IgG (heavy chain) conjugate was obtained from Microbiological Associates, Walkersville, MD. as a conjugated horseradish peroxidase IgG Fraction prepared in rabbits. The working peroxidase conjugate was prepared as a 1:400 dilution in the Tween NaCl diluent. Aliquots of the diluted working conjugate were stored at -20°C until use. Working conjugate, at room temperature, was delivered as a 5 lambda drop onto the slide antigen-serum spot, maintaining a 5mm diameter spot-surface contact, kept humidified at 37°C for 30 minutes, and followed by washing and drying as performed in the application of serum samples to the antigen spot. The conjugate dilution of 1:400 was determined by titration to be optimal. Substrate was a freshly prepared mixture of 9 parts, 80 mg% aq., of 5-Amino Salicylic acid (ICN Pharmaceutical Inc., Plainview, N.Y.) pH, adjusted to 6 with 1N NaOH and 1 part 0.05% H₂O₂ aq. (Mallinckrodt, Inc., St. Louis, MO.) added as an oxidizer (67). Filter paper (Whatman ML ashless #41) was cut to the same dimensions as the microscope slide, saturated with 500 lambda of the prepared substrate, and carefully overlaid on the slide. Production of the purple-brown substrate product is visually monitored at the control spots site for approximately 4 to 6 minutes, then the filter paper is removed and air dried. A test positive or negative serum is visually determined relative to the controls. A positive titer was defined to be a 3 to 4 fold dilution of serum. Positive and negative sera controls were tested on each slide.

RESULTS: The MIFA procedure and our peroxidase paper enzyme-linked immunosorbent assay (PELISA) using an enzyme conjugate were studied in ability to detect antibody in 351 human sera. An agreement of 96.3 percent with the MIFA procedure in the screening of antibody at a 1:50 serum dilution against a

Table 16 Comparative Screen (1:50 serum dilution) Agreement
and Disagreement Between the MIFA and ELISA Methods
for Detection of Serum Antibody to Scrub Typhus
Antigens (n = 351)

Study groups	Total sera	MIFA		ELISA	
		Positive	Negative	Positive	Negative
TP1	98	66	32	69	29
TP2	105	78	27	82	23
TP3	10	3	7	5	5
PC2	50	0	50	0	50
PC3	58	3	55	2	56
KLX	30	21	9	20	10

Disagreements

TP1	98		3	3	
TP2	105		4	4	
TP3	10		2	2	
PC2	50				
PC3	55	2	1	1	2
KLX	30	1			1

Totals

Disagreement		3	10	10	3
Agreement		168	170	168	170
Sera	351	171	180	178	173

=====

Agreement: 96.3% Disagreement: 3.7%

Antibody positive: ELISA greater than MIFA: 2%

Antibody negative: MIFA greater than ELISA: 2%

Table 17 Comparative Agreement and Disagreement Between the
MIFA and ELISA methods for the Detection of Serum
Antibody to Scrub Typhus Antigens.

Study groups	Pool 1:50 screen	Pool titer 1dil/2dil	Karp titer 1dil/2dil	Kato titer 1dil/2dil	Gilliam titer 1dil/2dil
TP1 agree	95	71/93	77/91	80/87	75/84
disagree	3	27/5	21/7	18/11	23/14
TP2 agree	101	80/97	84/97	86/98	81/98
disagree	4	25/8	21/8	19/7	24/7
TP3 agree	8	7/8	8/8	7/7	6/8
disagree	2	3/2	2/2	3/3	4/2
PC2 agree	50	50/50	50/50	50/50	50/50
disagree	-	-/-	-/-	-/-	-/-
PC3 agree	55	55/55	55/55	57/57	55/55
disagree	3	3/3	3/3	1/1	3/3
KLX agree	29	20/29	24/28	24/29	26/28
disagree	1	10/1	6/2	6/1	4/2
TOTAL agree	383	283/332	298/329	304/328	298/323
disagree	13	68/19	53/22	47/23	58/28
% agree	96.3	80.6/94.6	84.9/93.7	86.6/93.4	83.5/92.0
% disagree	3.7	19.4/5.4	15.1/6.3	13.4/6.6	16.5/8.0

The screening is performed against the three antigen pool at a
1:50 dilution. Results are expressed as in agreement or
disagreement within one or two serum dilutions between the methods.

Table 18 Enlarged photographs of 3 PELISA Results

positive: +, negative: -, serum control: C

		A	B	C	D
I.	1.	+C	+C	-C	-C
	2.	-C	+C	+C	+C
	3.	-C	-C	+C	+C
II.	1.	-	-	+	+
	2.	+	+	+C	-C
	3.	+	+	-C	-C
III.	1.	-	-	-	-
	2.	+	+	+	+
	3.	-	-	+C	-C

II/3/C and D are reagent, no antigen, normal yolk sac
controls tested against a positive and negative control
sera.

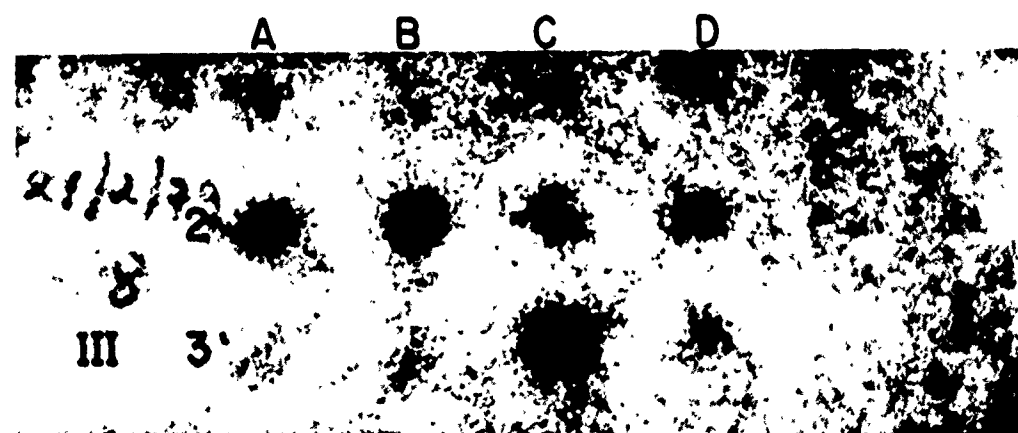
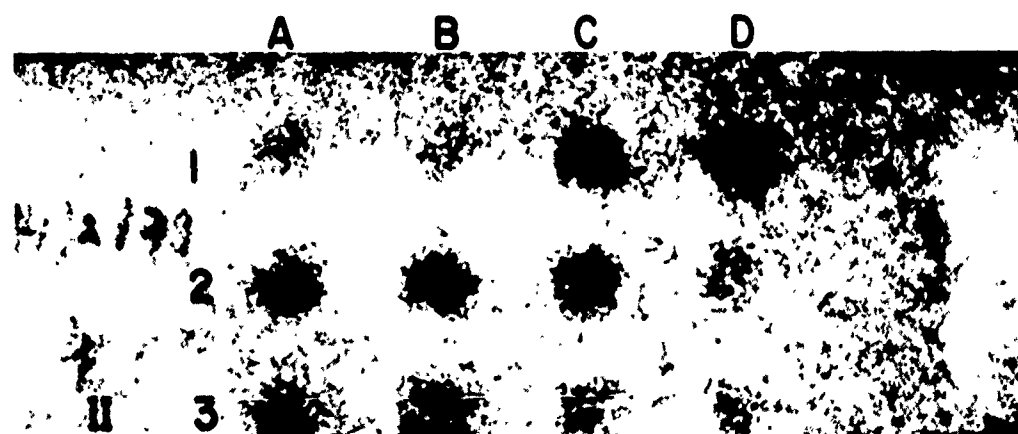
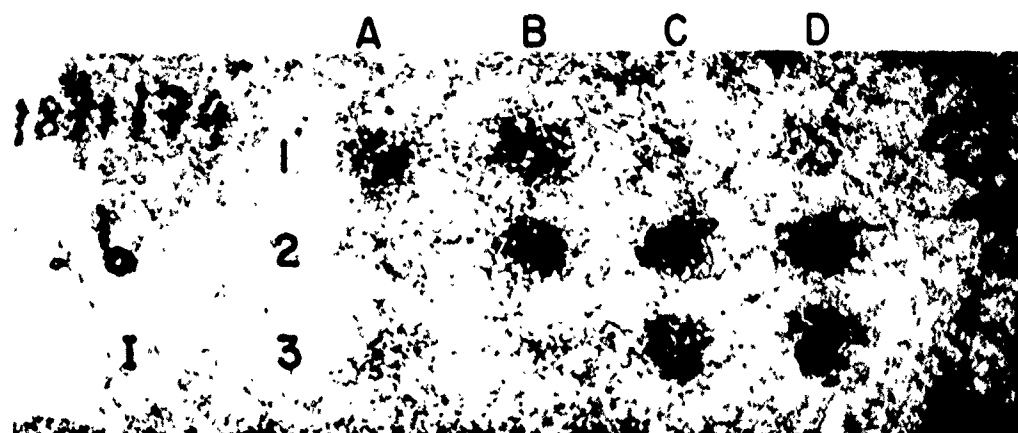


Figure 4

heterologous antigen pool of Karp, Kato and Gilliam strains of the scrub typhus rickettsia was obtained (Table 6). Of the sera screened positive for antibody, two percent more were detected by PELISA. Of negative sera, two percent more were detected by the MIFA than by PELISA.

In performing titers, by 2-fold serial dilutions of the serum, comparison was made as agreement or disagreement between the two methods as being within one or two dilutions (Table 17). Titer agreement with two dilutions was 94.6 percent for the pool, 93.7 percent for the Karp strain, 93.7 percent for Kato and 92.0 percent for Gilliam. These agreement percentages represent serum with detectable antibody as well as those without detectable antibody levels. Positive antibody detection ranged from the initial 1:50 dilution to 1:1600, all in 2-fold serial dilutions.

Figure 4 is an enlarged photograph and code description (Table 18) of the dry PELISA 75 x 22 mm microscope slide size filter paper strips. Strip I represents only positive and negative control sera with all except I/1/C, I/1/D, I/2/A, I/3/A and I/3/B being positive. Strip II represents actual test sera and four controls, II/2/C and II/2/D being a positive and negative control sera and II/3/C and II/3/D being uninfected no-antigen normal yolk sac reagent controls tested against a positive and negative control serum. Strip III represents tested sera and a positive and negative control sera at III/3/C and III/3/D.

10. Mosquito Cytogenetics, Electrophoresis and Cross Mating Studies

OBJECTIVE: To define and delimit the species or strains of mosquito species in Thailand and Southeast Asia that serve as the primary vectors of human pathogens by cytogenetic and enzymatic techniques for:

1. A check against current morphological species concepts;
2. the accurate determination of the limits of gene pools in natural populations of vector species; and
3. the correlation of genetic variation in natural and/or colony populations of the primary vectors with their degree of susceptibility to infection with dengue viruses and/or malaria parasites.

BACKGROUND: These studies are a continuation of projects initiated in 1978 and outlined in a previous report (23). The recognition of sibling or cryptic species in important vector species groups of mosquitoes is steadily increasing with each year, and is essential for developing effective control programs and understanding the epidemiology of vector-borne diseases (68). Cytogenetic and electrophoresis techniques have been shown to be effective in differentiating such cryptic species and in determining the genetic variability (including disease susceptibility) in natural populations of mosquitoes (69). These techniques, when coupled with morphological studies and hybridization experiments, provide the best basis for species and vector strain differentiation.

METHODS: Cytogenetic techniques employed were a modification of the standard chromosome squash technique (70) for salivary polytene chromosome preparations and a modified technique (71) for larval brain metaphase preparations. The electrophoresis techniques employed and the enzyme terminology and abbreviations are those of Steiner and Joslyn (72).

Chromosome maps and electrophoresis starch-gel esterase patterns are being made for selected laboratory colony strains or species. These maps or patterns will serve as 'standards' for later comparisons with other strains and/or species. Once "standard" maps or esterase patterns have been established, wild collected populations will be sampled to survey the variations occurring in natural populations. Wild and/or colony strains of currently recognized morphological species that exhibit sufficient cytogenetic and/or electrophoretic differences will be studied further by hybridization experiments to determine if they are conspecific. Strains of Aedes aegypti (wild or colony) that exhibit distinct esterase polymorphisms will be tested for susceptibility to infection with dengue viruses.

RESULTS: Two additional colonies of members of the Leucosphyrus Group were established during this period. A colony of the Taiwan strain of An. balabacensis was started with the assistance of Dr. J.C. Lien, Taipei, and the U.S. Navy Medical Research Unit No. 2, Taipei, Taiwan. This colony requires forced mating and most females require two blood meals before oviposition. The second colony was acquired from Mr. W.H. Cheong, Institute of Medical Research, Kuala Lumpur, Malaysia. This colony purportedly represents balabacensis from an inland locality in Sabah, East Malaysia; however, after a very short time the colony was noted to be self-mating, and a morphological examination of the adult and immature stages showed the colony was the Perlis-Kedah strain

(

(Peninsular Malaysia) of balabacensis. This identification was confirmed by personnel at the Medical Entomology Project (MEP), Smithsonian Institution, Washington, D.C.

Efforts to colonize other vector species or potential vectors from Thailand are continuing. Two colonies of An. maculatus (Central and Southeast Thai strains) have been started, however, these colonies are still unstable. Efforts are continuing to colonize nivipes and/or philippinensis from Thailand for a number of studies.

During this report period, the Thai species called balabacensis and previously listed (23) as a colony used in these studies was involved in a taxonomic study which resulted in its being described as a new species, dirus, in the Leucosphyrus group from Thailand (73). A more detailed discussion of this new species is provided elsewhere in this Annual Progress Report. This species, dirus, is one of the primary species involved in cytogenetic and electrophoresis studies.

Electrophoretic studies on An. maculatus were initiated during the year. This species is considered a primary vector of human malaria parasites in Thailand. However, its vector capabilities in the southern peninsular area appear to be different from those in the central and northern areas of Thailand. During the last half of 1978, a large number of adult female maculatus were collected in Chumphon Province. Table 19 shows the genetic variation occurring in Chumphon females screened at nine different electrophoretic enzyme loci. These specimens were screened to help determine the enzyme loci showing allelic variation that can be used in future studies. Actually, no analysis of the Chumphon maculatus electrophoretic esterase banding patterns is possible at this time. Chumphon Province probably represents the most northern extension of many Malaysian species in Thailand, thus a number of species, subspecies and other taxa probably overlap in that area. Work is continuing on maculatus strains, but, rests, in part, on the establishment of a successful maculatus colony from central-northern Thailand.

Electrophoresis studies were also initiated during this period on member of the Leucosphyrus Group. A total of 12 different enzyme loci were screened for genetic variation in colony adults of An. dirus and nine were screened in colony adults of the Perlis strain of balabacensis (Table 20). The enzyme loci Hk-1, Me and 6-pgdh were homozygous in these studies, however, larger sample

sizes will be used in future studies. Considerable allelic variation was detected in the enzyme loci Aldox, Est-2 and Xdh, and differences were detected between dirus and the Perlis strain in frequencies of certain alleles at several enzyme loci. Analyses of these variations and differences are continuing and future comparisons will include the Taiwan strain of balabacensis.

A total of 15 enzyme loci were screened for genetic variation in 50 larvae each of four colony strains of Thai Aedes aegypti (23). Only six enzyme loci exhibited allele variation in electrophoretic esterase banding patterns during this study (Table 21). Differences were detected in the aegypti strains, particularly at the loci Pgm, Est-1 and Est-2; however further studies and analysis are necessary for proper interpretation of these differences. Further electrophoretic analyses of the aegypti strains are anticipated, coupled with an analysis of each strain's susceptibility to dengue viruses 1-4.

The frequencies of alleles for the enzyme loci, Esterase 1, 2, 3, 4 and Lap 1, 2, 3, 4 in 4 strains of 4th stage larvae of aegypti reared at 20° and 35°C are presented in Table 22. The most marked differences were noted in the allele variation at the Est 1, 2, 3 and 4 loci for all four strains of aegypti. Larvae reared at 20°C exhibited striking allele frequency differences at all 4 Est loci, particularly the differences between the Din Daeng F₁ larvae and colonized strains 1, 3 and colonized strains 1, 3 and 4. Differences in allele frequencies at the lap 1, 2, 3 and 4 loci were observed for all four strains of aegypti, but were considerably less than those observed for the Est loci. These data indicate that the four strains of aegypti not only show interstrain differences in allele frequency at a given temperature, but also show intrastrain allele variations when reared at different temperature.

Cytogenetic studies during this period focused on members of the Anopheles Leucosphyrus Group. A limited number of squash preparations were made from adult female ovarian nurse cells; however this technique did not yield slide preparations of the same quality as salivary gland polytene chromosome preparations. Excellent salivary gland squash preparations have been made for Anopheles dirus (Figure 5) and An. balabacensis (Perlis strain). Brain metaphase slides have also been prepared from adult specimens of these two colonies. Both the salivary and brain metaphase chromosome preparations are currently being analyzed in preparation for publication. A salivary chromosome map has been prepared for dirus and is included in a manuscript being

Table 19 Genetic variation (allele frequencies) at 9 different electrophoretic enzyme loci in feral Anopheles maculatus females from Chumphon Province, Thailand (1978)

Enzyme locus	Populations ⁽¹⁾	No. ♀♀	Allele				
			.98	1.00	1.02	1.04	1.06
6-pgdh	07970, 08007 07945, 08003 08004	53	-	.85	.11	.04	-
Hk-1	08003, 08004	5	-	1.00	-	-	-
Hk-3	07945, 08004	11	-	1.00	-	-	-
Xdh	08003, 08007 08003	68	.01	.71	.13	.06	.09
α-gpdh	07970, 08007 07945, 08003	42	-	1.00	-	-	-
Idh	08003, 08004	39	.15	.81	.04	-	-
Aldox	08003, 08004	30	-	-	.97	-	.03
Pgi	08003	29	-	-	-	1.00	
Pqm	08003	29	-	.04	.94	.02 ⁽²⁾	

(1)

AFRIMS collection numbers for wild females taken in biting collections on human bait.

(2)

Further analysis needed.

Table 20 Presence of genetic (allelic) variation at 12 different electrophoretic enzyme loci in adults from colonies of Anopheles dirus and balabacensis (Perlis Strain) at AFRIMS, Bangkok.

Enzyme locus	Presence (+) or absence (-) of genetic variation							
	<u>Anopheles dirus</u>				<u>An.balabacensis</u> (Perlis strain)			
	♂	No. Tested	♀	No. Tested	♂	No. Tested	♀	No. Tested
Aldox	+	163	+	202	+	129	+	134
Est-1	-	45	-	103	—	Not Tested	—	—
Est-2	+	45	+	103	—	Not Tested	—	—
Est-3	+	30	Not Tested		—	Not Tested	—	—
Hk -1	-	58	-	48	-	15	-	15
Hk -2	-	58	+	48	-	39	+	38
Hk -3	+	81	-	81	-	15	-	15
Me	-	47	-	30	-	68	-	81
Pgi	-	130	+	219	-	114	+	88
Pqm	+	147	+	194	-	15	-	15
Xdh	+	100	+	144	-	65	+	62
6-pgdh	-	84	-	94	-	58	-	78

Table 21 Genetic variation (allele frequencies) at 6 different electrophoretic enzyme loci in 4th stage larvae of 4 colony strains of *Aedes aegypti* from Thailand (AFRIMS-Bangkok).¹

Enzyme locus	Mosquito Strain	Allele				
		.96	.98	1.00	1.02	1.04
Mdh	<u>aegypti</u> -1	-	.17	.72	.11	-
	" -3	-	.20	.80	-	-
	" -4	-	.20	.75	.05	-
	" -5	-	.10	.80	.10	-
Pgm	<u>aegypti</u> -1	-	-	.50	.40	.10
	" -3	-	.09	.50	.32	.09
	" -4	-	-	.50	-	.50
	" -5	-	-	.60	.30	.10
Idh	<u>aegypti</u> -1	-	-	1.00	-	-
	" 3	-	-	.91	.09	-
	" 4	-	-	1.00	-	-
	" 5	Not Analyzed				
Est-1	<u>aegypti</u> -1	-	-	.90	.10	-
	" -3	-	-	.80	.20	-
	" -4	-	.25	.70	.05	-
	" -5	.20	.30	.20	.30	-
Est-2	<u>aegypti</u> -1	-	1.00	-	(?) Null ² (?)	
	" -3	-	-	1.00	(?) Null (?)	
	" -4	-	-	-	1.00 Null (?)	
	" -5	Not Analyzed				
Pgi	<u>aegypti</u> -1	-	-	1.00	-	-
	" -3	-	-	1.00	-	-
	" -4	-	-	1.00	-	-
	" -5	-	.10	.90	-	-

¹ The following 9 enzyme loci did not exhibit genetic variation during these tests (α -G-pdh, G-6-pdh-F, Ald-M, Ald-F, 6-pgdh, Got-S, Got-F, Me and Lap-F).

² Null (?) - possible overlap in activity between the Est-1 and Est-2 loci

Table 22 Relative allele frequencies of esterase and lap enzyme loci in 4th stage larvae of 4 strains¹ of *Aedes aegypti* reared at 20° C and 35° C. (AFRIMS, Bangkok)

Locus	<i>Aedes aegypti</i> Strains	No. of Larvae	Allele (20° C)			No. of Larvae	Allele (35° C)		
			.98	1.00	1.02		.98	1.00	1.02
Est-1	aegypti-1	28	.07	.63	.30	23	.16	.61	.23
	aegypti-3	16	-	.45	.55	23	.11	.89	-
	aegypti-4	26	-	.88	.12	00	ND ²	ND	ND
	aegypti-D.D	08	.06	.50	.44	08	.05	.25	.69
Est-2	aegypti-1	23	.10	.83	.07	23	-	1.00	-
	aegypti-3	16	.07	.76	.17	27	.02	.98	-
	aegypti-4	26	.09	.81	.10	00	ND	ND	ND
	aegypti-D.D.	08	.13	.68	?	08	-	100	-
Est-3	aegypti-1	23	-	.90	.10	23	-	blurry	-
	aegypti-3	21	-	.91	.09	24	-	1.00	-
	aegypti-4	21	-	1.00	-	00	ND	ND	ND
	aegypti-D.D.	03	-	1.00	-	03	-	1.00	-
Est-4	aegypti-1	08	.06	.94	-	23	-	1.00	-
	aegypti-3	08	.06	.94	-	24	-	1.00	-
	aegypti-4	26	-	1.00	-	00	ND	ND	ND
	aegypti-D.D.	08	.13	.87	-	08	-	1.00	-
Lap-1	aegypti-1	28	.05	.95	-	23	-	1.00	-
	aegypti-3	16	.04	.96	-	24	-	1.00	-
	aegypti-4	18	-	1.00	-	25	-	1.00	-
	aegypti-D.D.	08	-	1.00	-	08	.13	.87	-

Table 22 Continued

Locus	<i>Aedes aegypti</i> Strains	No. of Larvae	Allele (20°C)			No. of Larvae	Allele (35°C)		
			.98	1.00	1.02		.98	1.00	1.02
Lap-2	aegypti-1	28	-	.94	.06	23	.42	.46	.12
	aegypti-3	11	-	1.00	-	00	ND	ND	ND
	aegypti-4	18	-	.76	.24	00	ND	ND	ND
	aegypti-D.D.	10	-	1.00	-	10	-	.93	.07
Lap-3	aegypti-1	28	-	1.00	-	00	ND	ND	ND
	aegypti-3	16	-	1.00	-	00	ND	ND	ND
	aegypti-4	18	-	1.00	-	00	ND	ND	ND
	aegypti-D.D.	08	-	1.00	-	08	-	1.00	-
Lap-4	aegypti-1	23	-	1.00	-	00	ND	ND	ND
	aegypti-3	08	-	1.00	-	00	ND	ND	ND
	aegypti-4	13	-	.92	.08	00	ND	ND	ND
	aegypti-D.D.	03	-	1.00	-	03	-	1.00	-

1 Generation: Colony 1 (unknown-11 yrs old), Colony 3 (F₁₉),
 Colony 4 (F₁₀) and Din Daeng (F₁)

2 Not done



Figure 5 Salivary polytene chromosomes of Anopheles dirus Peyton and Harrison.

prepared for publication in the near future. The specimens of dirus used to prepared this map came from a colony which is approximately 15½ years old, and as would be expected, polymorphisms were not observed on the chromosomes. The chromosome map for dirus will serve as a "standard" for comparisons with future maps of other taxa in the Leucosphyrus Group. Recently, squash preparations have been started for the Taiwan strain of balabacensis and a future comparison of the chromosomes of this strain with those of the Perlis strain and dirus is anticipated.

Cross mating experiments between An. dirus and An. balabacensis (Taiwan strain) are currently in progress. Crossing experiments between dirus and balabacensis (Perlis strain), and between the Taiwan and Perlis strains will start in the near future.

All aspects of these studies are continuing.

11. Mosquito Survey and Taxonomic Studies

OBJECTIVE: To elucidate the mosquito fauna of Thailand and Southeast Asia, with primary emphasis on the identification of diagnostic characters for the separation of vector species and groups containing vector species of human pathogens.

BACKGROUND: This is a continuation of efforts begun in the early 1960's and currently conducted in partial collaboration with the Medical Entomology Project (MEP), Smithsonian Institution, Washington, D.C. A brief review of the history of these efforts was presented in the previous annual report (23).

METHODS: Surveys for target species and species groups are conducted at selected sites and habitats throughout Thailand. These surveys involve the collection of immature and adult mosquitoes, with emphasis placed on reared adults with associated larval and pupal skins, and on progeny adults (with associated larval and pupal skins) reared from eggs oviposited by known wild collected females. Specimens are curated and pinned or slide mounted for further study by investigators in the laboratory, or shipped for study to MEP or other world recognized authorities. Studies primarily consist of an analysis of intra-interspecific variations to identify useful characters for separating the species. Useful diagnostic characters, new species records, and new taxa found in Thailand are prepared for publication and described in scientific journals.

RESULTS: During this period major research efforts went into support for the monographic revisionary study of the Leucosphyrus Group of Anopheles (Cellia) by personnel at the Medical Entomology Project (MEP), Smithsonian Institution, Washington, D.C. These collaborative efforts are responsible for the recent discovery that the primary malaria vector species in Thailand, previously called balabacensis, is morphologically distinct in the adult, pupal and 4th larval stage from topotypic balabacensis from Balabac Island, Philippines, and specimens of this species from the adjacent areas of Palawan Island, Philippines and Sabah, East Malaysia. Accordingly, the Thai species has been redescribed as a new species, Anopheles dirus (73). Historically, a number of major malaria-vector efforts in Thailand (e.g. 74, 75) have been based on dirus (as balabacensis). In addition, dirus has been colonized since 1964 and is known around the world as the Khao Mai Phaeo or Bangkok Strain (of balabacensis), and has been used for years in numerous experimental malaria research projects and scientific publications. Current ongoing research with this species consists of: (A) cross mating experiments with strains of balabacensis sensu lato, from Southeast Asia; (B) continuing taxonomic studies; and (C) cytogenetic and electrophoresis studies (23, and this Annual Progress Report).

Field and laboratory support for the Leucosphyrus Group studies resulted in the shipment of 1,898 adults and 1,786 slides of whole larvae and larval and/or pupal skins of the above adults to the Medical Entomology Project, Smithsonian Institution. Approximately half of these specimens were progeny broods from laboratory colonies, while the remainder consisted of reared feral adults (with associated immature skins) and progeny broods from feral females from several areas of Thailand. These specimens are being analyzed for intra-interspecific variations, with emphasis on the discovery and confirmation of reliable key characters for differentiating the taxa in this group. Biological and behavioral data from the laboratory and field collections are being analyzed for additional information of potential use in differentiating the various taxa being studied.

Several very capable vectors of human malarial parasites in the Indian subregion, that are uncommon or rare in Thailand, were collected during this period. Anopheles stephensi and varuna were collected as larvae (and reared) from stream pools in Hang Dong District, Chiangmai Province. Anopheles culicifacies larvae were found common in the Mae Klong river (Khwaie Yai) just 12 km upstream from Kanchanaburi. The first two species are very uncommon and known only from the northwest corner of Thailand.

However, culicifacies is more widespread and is possibly becoming more abundant. Since culicifacies has developed DDT resistance in northern Thailand (76), its distribution and abundance deserve close watch.

A monographic study of the Anopheles Myzomyia Series in Thailand, including aconitus, culicifacies, jeyporiensis, minimus, pampanai and varuna, has been completed and submitted for publication (77). Both aconitus and minimus are confirmed vectors of human malarial parasites in Thailand. This study summarizes nearly three years of field work in Thailand and over three years of laboratory-museum work on more than 36,000 specimens.

Anopheles campestris, an established vector of human malarial parasites in Malaysia and a potential vector in Thailand, has been recorded from the Chiangmai Valley in northern Thailand on several occasions. A series of 247 adults of the Barbirostris Group, reared with 4th stage larval and/or pupal skins from collections in five districts in and adjacent to the valley, was recently examined for campestris. Although most adults were identifiable as barbirostris some possessed key characters identifying them as campestris, while others were intermediate. Fortunately, the pupal stage contains the best diagnostic characters for separating these two species in Thailand (78), and the pupae were clearly all barbirostris. All of the 4th stage larval skins that were examined were also barbirostris. Accordingly, previous records of campestris in Chiangmai should be disregarded, and probably were based on adult variants of barbirostris.

In certain sections of eastern Indonesia, human malarial and filarial parasites (Brugia malayi and B. timori) are transmitted by Anopheles barbirostris, which in the remainder of its wide distribution is a poor or non-vector. These vectorial and certain morphological differences have been used in the past to justify the description of a subspecies, innominata, for the vector strain of barbirostris (79). The possibility of another sibling species in the Barbirostris Group recently prompted a morphological re-examination of specimens from eastern Indonesia, Malaysia, Thailand and other areas. This collaborative effort concluded that specimens of the vector strain from the islands of Sulawesi, Flores and Timor were conspecific with barbirostris from Java, Malaysia, Thailand and elsewhere (80). Adults of the vector strain were found more variable, however the immature stages were all clearly barbirostris. The variations noted were considered intraspecific and it was concluded that the recognition of the subspecific name, innominata, is not warranted.

Anopheles maculatus is currently recognized by the Malaria Division, Thai Ministry of Public Health, as a primary vector of human malarial parasites in Thailand. However, maculatus is apparently a vector only (or primarily) in southern Thailand near the Malaysian border. A critical morphological re-examination of this species is urgently needed in Thailand and adjacent areas of Southeast Asia. In preparation for such studies, hundreds of adults of maculatus have been reared with associated immature skins from Chiangmai, Chumphon and Phangnga provinces and collections are continuing. A previously unreported melanistic mutant of maculatus has also been described with notes on the inheritance of this trait (81).

The revisionary study on the Aedes (Finlaya) kochi group of species in Thailand and Malaysia (23) made significant progress during this period. Over 1,800 specimens (adults and immature slides) of the Poicilius Subgroup and approximately 150 adults and immatures of the Flavipennis Subgroup were collected in Thailand and curated for future study. Currently, four species in the Kochi Group are recognized from this study area. Reliable diagnostic characters have been found for the adults and immatures of these species. Descriptions and illustrations are being prepared for publication.

In the previous Annual Progress Report (23), a large series of specimens from Kanchanaburi Province was noted as shipped to the Medical Entomology Project, Smithsonian Institution. These specimens were recently described as a new Aedes subgenus, Isoaedes, and a new species, cavaticus (82). This new taxon is apparently a true cave dwelling species known only from Gang Lawa Cave, Huai Bong Ti, in Kanchanaburi Province.

Two training courses were offered during the year. The first, "Curatorial Techniques for Mosquito Specimens", was conducted during the period 19-27 March 1979, and four participants from the Thai Ministry of Public Health attended. The second course, "Entomological Field and Laboratory Techniques for Malaria Surveillance", was offered during the period 2 July to 17 August 1979, and was attended by two Royal Thai Navy personnel.

These studies are continuing.

12. Studies on Filariasis in Small Mammals in Northeast Thailand

OBJECTIVE: To establish a consistently reproducible, readily available, genetically controlled laboratory animal model for the study of infection with nematodes of the Superfamily Filariidae.

BACKGROUND: The need for a consistently reproducible laboratory animal model for studies of infection with nematodes of the Superfamily Filariidae has been expressed (83). A mosquito-transmitted filaria-laboratory rat system would fulfill this requirement and is the ultimate goal of these studies. Attempts to transmit Brugia tupaia through mosquitoes to various laboratory animals were made at the SEATO Medical Research Laboratory (SMRL) in 1969 and 1970 (84, 85) but were unsuccessful. One mosquito-transmitted filarial parasite, Brientalia booliati, has been reported in Malaysia and laboratory rats have been successfully infected with this nematode at the University of Singapore (86, 87). Additionally, one report of mosquito transmission of Brugia pahangi to mice using Aedes togoi at the University of Singapore is reported (88).

A preliminary study in which 1,694 wild rodents in Thailand were trapped and screened for microfilaria was undertaken by Dill, et al. (89). Results of this study revealed the presence of unreported filarial nematodes of several species. Mosquito transmission studies utilizing these new species of microfilaria were unsuccessful. Subsequent to this study, one of the new species of microfilaria has been described and named Dunnifilaria dilli (90).

This study is modeled after the earlier study by Dill (89) et al.

METHODS: Small mammals were live-trapped in the area of Pak Chong, using bananas as bait. The animals were screened in the field by examining blood films for the presence of microfilaria. Blood was obtained by either orbital bleeding or by cardiac puncture. The number of each species trapped and the number positive for microfilaria (regardless of species) is seen in Table 23.

Thirty-three of the positive animals were transported to our laboratory for definitive identification of the microfilaria and further studies. Four animals died either during the trip to the

Table 23

<u>Species</u>	<u>Number Caught</u>	<u>Number Positive</u>
Bandicota savilei	3	0
Hylomys suelis	10	0
Menetes berdmorei	26	3
Menetes cerviollor	15	0
Rattus berdmorei	1	0
Rattus bukit bukit	33	0
Rattus exulans	2	0
Rattus koratensis	19	5
Rattus losea	1	0
Rattus rattus	153	5
Rattus rapid orbus	1	0
Rattus sabanus	37	19
Rattus surifer	159	7
Tupaia glis	<u>90</u>	<u>25</u>
Total	550 =====	64 =====

Table 24

Animal Species	Microfilaria Species			
	Dunnifilaria ramachandrani	Dunnifilaria dilli	Orugia tupaia	Brienlia sergenti
Rattus sabanus*	10	4	0	0
Rattus surifer	0	3	0	0
Rattus koratensis*	4	2	0	0
Rattus rattus	1	0	0	0
Menetes berdmorei	0	0	0	1
Tupaia glis	0	0	6	0

* Total is more than 100% because both species of microfilaria present in the same animal in some cases.

Table 25

Microfilaria Count 10^3 /ml. in Rodent Blood

Animal No.	Microfilaria Species	Time Recorded											
		8.00	10.00	12.00	14.00	16.00	18.00	20.00	22.00	24.00	02.00	04.00	06.00
791	D. ramachandran	564	0.2	524.6	408.4	580	177	396	550	500	270	569	560
628	D. ramachandran	229.6	212.6	463	477.4	437.2	475.2	427.8	624	471.6	204.4	459	354.8
741	D. ramachandran	5.6	4.2	8.0	8.8	8.6	11.5	10.8	4.8	11.6	21.0	13.0	1.2
538	D. ramachandran	258	206	344.4	249.6	280.8	411.4	375.4	75.4	39.0	240.8	419	382
427	D. ramachandran	99.6	156	64.4	178.2	121.4	56.8	154	56	157.2	157	151.6	77.4
628	D. dilli	93.6	168.6	314.4	316.4	284.4	332.4	315.6	397.4	346.2	156.8	316.4	303.6
427	D. dilli	41	69	38.8	75.6	53.6	26.4	67.6	27.2	45.4	56	55.6	49.8
538	D. dilli	115.8	99	154.2	130.2	147	182.2	185.8	11.2	18	28.6	48	55
484	D. dilli	12.6	19	20.2	31.2	9.8	19.2	11.2	11.2	17	5.4	10.2	11.2
772	B. sergentic	0.2	0.4	0.4	0.4	02.	0.2	0	0	0	0	0	0.2
331	B. tupaia	0	0	0	0	0	0	0	0.8	0.2	0.2	0	0
522	B. tupaia	0	359.4	0.2	0.2	0.8	0	0.2	0	0	0	0	0

Animal No. Species

791	Rattus koratensis
628	Rattus koratensis
741	Rattus rattus
538	Rattus sabanus
427	Rattus sabanus
484	Rattus surifer
772	Menetes berdmorei
331	Tupaia glis
522	Tupaia glis

laboratory or soon after arrival. Blood smears from twenty-nine of the animals were sent to Dr. Mak Joon Wah for identification of the microfilaria. The results of these identifications are seen in Table 24.

Periodicity studies were undertaken to determine the optimum time to feed mosquitoes for transmission studies. Microfilaria counts were performed every two hours for twenty-four hours using the method of Ramachandran (91). The following species of animals (with the number of animals of each species in parentheses) were used in the periodicity studies: Rattus koratensis (2), Rattus rattus (1), Rattus sabanus (2), Rattus surifer (1), Menetes berdmorei (1), Tupaia glis (2).

RESULTS: Results of the microfilaria counts of the periodicity studies are seen in Table 25. Number of animals used in this study are not sufficient to establish a definite cyclic pattern. Periodicity studies involving more animals will be conducted in the coming year.

Efforts in the future will concentrate on Dunnifilaria dilli and Dunnifilaria ramachandrani. Studies will be directed at description of the life cycle of these filarids and trials using initially Aedes togoi and Aedes albopictus for mosquito infectivity and transmission studies.

13. Studies on Canine Viral Enteritis

OBJECTIVES:

1. To identify and describe the etiologic agent producing a severe, bloody, often fatal diarrheal viral disease of canines in the military working dogs at the Royal Thai Army National War Dog Center in Pak Chong and the Royal Thai Navy Military Dog units at Sattaheep.

2. To produce Canine Viral Enteritis in susceptible, weanling dogs.

BACKGROUND: Parvoviruses are the smallest known viruses isolated from humans or animals. They have an affinity for rapidly replicating cells such as intestinal epithelial crypt cells and bone marrow. A parvovirus is the known etiologic agent of feline panleukopenia, a severe, often fatal disease of cats in which the epithelium of the intestinal tract in general and the crypt cells

in particular are destroyed, producing fatal hemorrhagic diarrhea. Binn, et al, first isolated and characterized viral agents from the feces of asymptomatic dogs in 1970 (92). These agents, referred to as Minute Virus of Canine measured 20 to 21 nm in diameter by electron microscopy, were present in the nuclei of infected cells where they sometimes produced intranuclear inclusions, were resistant to ether, chloroform, and heat treatment, were inhibited in their growth by 5-iodo-2-deoxyuridine, and caused hemagglutination of rhesus red blood cells at 5°C. These properties are consistent with membership in the parvovirus or picornavirus group.

The first report of parvoviruses being associated with diarrhea in young dogs was published in 1977 (93). Parvoviruses have been associated with enteric disease in several species including cats, rabbits, rodents, and calves (94, 95, 96, 97, 98). During 1978, several outbreaks of a severe, hemorrhagic diarrheal syndrome were reported from dogs (99, 100). In each incidence a parvovirus or parvo-like viral agent was demonstrated by electron microscopy.

During 1979, a severe, often fatal, hemorrhagic diarrhea began to appear in dogs in Thailand. The disease was particularly severe in areas where there were large numbers of dogs in close contact, i.e., the larger cities, veterinary hospitals, commercial kennels, and military and police dog training centers. The Royal Thai Army National War Dog Center in Pak Chong, Nakorn Ratchasima experienced a heavy death loss in their military working dogs due to enteritis from January to June 1979 (Table 26). Of the total deaths occurring during this period, 49 percent were due to some form of enteritis. Thirty-five of the deaths were the result of the same clinical syndrome of vomiting, rapid dehydration, and hemorrhagic diarrhea. No treatment appeared to alter the course of the disease. Simultaneously, several dogs stationed with the Royal Thai Navy at Sattaheep were stricken with an identical illness. In addition, numerous reports of a fatal diarrheal disease in privately-owned dogs were being received from private practitioners in Bangkok and from the School of Veterinary Medicine, Chulalongkorn University.

METHODS: In June, 1979, a team of AFRIMS investigators visited the Royal Thai Army National War Dog Center in Pak Chong for the purpose of obtaining specimens of blood and stool from acutely ill dogs in an attempt to isolate and identify the etiologic agent

responsible for this fatal diarrheal syndrome. Serum and stool samples were obtained from dogs on five separate trips to Pak Chong and three trips to the Naval Station at Sattaheep (Table 27). In addition, several serum and stool specimens were collected from dogs presented to the small animal hospital at the School of Veterinary Medicine, Chulalongkorn University, Bangkok, Thailand and from private veterinary clinics in the city of Bangkok, Table 28.

The second phase of this study involved attempts to reproduce canine viral enteritis in susceptible weanlings puppies.

Source of Parvovirus:

One of the stool specimens from one of the dogs (#9717) at the Royal Thai Army National War Dog Center was positive for parvo-like virus by electron microscopy and served as our source of parvovirus. A two percent suspension (Vol/vol) of this virus containing stool was buffered with one percent phosphate buffer solution (pH 7.2) containing one percent Bovine Serum Albumin and filtered through a 0.45 U millipore filter. This served as the inoculum used to infect the susceptible weanling dogs. A second stool from a dog (#4521) positive for both a parvo-like virus and an adenovirus was prepared in an identical manner and given to a second group of susceptible weanling puppies. One puppy from each group was given only phosphate buffered saline and served as a control. The infected stool suspension was given orally in each instance.

Susceptible Weanling Dogs:

Twelve, weaned, susceptible puppies approximately two months old were obtained from the municipal dog pound, Din Daeng Road, Bangkok. Six of the puppies were given anthelmintics to remove intestinal nematodes while six puppies were left unwormed. Reports in the literature indicate that intestinal parasites and pathogenic bacteria affect the severity of the disease so one group was left unconditioned (100). Complete blood counts and preinfection blood and stool specimens were collected for baseline data on all dogs. Serum was checked for the presence of anti-parvovirus antibodies. Stools were checked for pathogenic bacteria or protozoa. Stools and blood for antibody titer and virus isolation were collected starting at day 4 and periodically during the study according to Table 29. Other parameters such as body temperature, body weight, vomiting, diarrhea, and appetite were also followed during the course of the study according to the schedule in Table 29.

Table 26

Month	Jan	Feb	Mar	Apr	May	Jun	Total
No. Deaths	5	32	14	5	7	22	85
No, Enteritis	0	15	10	1	4	12	42

Table 27 Summary of Trips Made and Types of Specimens Collected

Trip Number	Date 1979	Location	Specimen
1	28 June	Pak Chong, National War Dog Center	Serum and stool
2	7 July	Pak Chong, National War Dog Center	Serum and stool
3	26 July	Pak Chong, National War Dog Center	Serum
4	7 August	Pak Chong, National War Dog Center	Necropsy
5	9 August	Pak Chong, National War Dog Center	Serum
6	30 July	Naval Station, Sattaheep	Serum
7	2 August	Naval Station, Sattaheep	Serum and stool
8	14 August	Naval Station, Sattaheep	Serum

Table 28 Summary of Specimens Collected

Study Location	Number of Dogs Sampled	Number of Samples		
		Paired Serum	Stool	Necropsy
NWD-P	27	26	8	1
NS-S	50	36	12	0
CU-VH	38	5	5	0
PC-B	8	3	5	1

NWD - National War Dog Center, Pak Chong

NS-S - Naval Station, Sattaheep

CU-VH - Chulalongkorn University, Veterinary Hospital

PC-B - Private Clinic, Bangkok.

Table 29 Schedule of Laboratory and Clinical Observations.

1979 Day/ Month	Day	Clinical Observations					Laboratory Work					Remarks
		Temp.	Wt.	Eat	Vomition	Diarrhea	** CBC	Serum	Stool Virus Isolation	Stool Culture	Fecal Exam.	
6-8	-4	X	X				X	X	X	X	X	
7-8	-3	X	X				X		X			
8-8	-2	X					X		X	X	X	
9-8	-1	X					X	X	X			
10-8	0	X					X		X	X	X	
11-8	+1	X					X		X			
12-8	+2	X					X		X			
13-8	+3	X	X				X		X			
14-8	+4	X					X		X			
15-8	+5	X					X		X			
16-8	+6	X					X		X			
17-8	+7	X					X	X	X			
18-8	+8	X					X		X			
19-8	+9	X					X		X			
20-8	+10	X	X				X		X			
21-8	+11	X					X		X			
22-8	+12	X					X		X			
23-8	+13	X					X		X			
24-8	+14	X					X	X	X			
27-8	+17		X									
31-8	+21							X				
3-9	+24		X									
7-9	+28							X				
10-9	+31		X									
14-9	+35							X				
17-9	+38		X									
21-9	+42							X				*

* Sacrifice & Necropsy, Terminate the project.

** Hct, WBC, Differential Count.

A complete necropsy was performed on all animals that died during the study. Specimens from the ileum, jejunum, liver, lung, kidney, spleen and stool were frozen at -70°C and saved for virus isolation. Likewise specimens from the ileum, jejunum, lung, liver, kidney, spleen were placed in 2.5 percent glutaraldehyde, processed in the usual manner and examined for the presence of parvo-like virus particles by the electron microscope. Tissues from all organ systems were placed in ten percent buffered formalin and processed in the usual manner for light microscopy. Dogs still alive at day +42 of the study were euthanized and their tissues processed as stated above.

RESULTS: Two of fourteen stools collected from military working dogs at the Pak Chong War Dog Center were positive for parvo-like virus particles by electron microscopy (#9717 and #4521). The stool from dog #4521 also was positive for adenovirus particles.

One hundred and fifty seven serum specimens from seventy-five dogs and twenty-four stool specimens from twenty-four dogs were shipped to Dr. L.N. Binn. Results of the examination of these specimens for the presence of antibody or virus are not yet available.

Results of the study designed to experimentally reproduce Canine Viral Enteritis in susceptible puppies are pending the analysis of the data.

14. Ecology and Epidemiology of Dengue Viruses in Din Daeng District, Bangkok

OBJECTIVES:

1. To determine the seasonal incidence of apparent and inapparent dengue virus infections in Bangkok adults and children.
2. To establish the duration and magnitude of the antibody response to primary and secondary arbovirus infection, including dengue virus type 1, 2, 3, 4 and Chikungunya virus.
3. To assess experimentally the ability of wild Ae. aegypti to serve as a vector of dengue viruses on a seasonal basis.
4. To determine the population density of the wild Ae. aegypti population on a seasonal basis and seasonal availability and the utilization of artificial containers by this species for oviposition.

BACKGROUND: Epidemiological and ecological investigations have shown that dengue viruses are endemic in Bangkok and that the primary vector and vertebrate host are Aedes aegypti and man, respectively (101, 102). Apparent human infections occur throughout the year; however, a marked increase in the incidence of illness has been observed during the rainy season (June-September). Age-specific attack rate data have placed children 15 years of age or less at greater risk (102). Early findings indicated that the variation in the incidence of infection was related to the population density of Ae. aegypti (103). More recent data suggest that the magnitude of change in absolute population size of Ae. aegypti in Bangkok was not large enough to explain the seasonal fluctuations in the incidence of apparent dengue virus infection (104). The latter investigation was extended to consider the longevity and blood feeding patterns of aegypti. Data did not show seasonal variation in 24 hour survival; however, biting rates appeared to vary as indicated by a marked decrease during the cool season of the year (105). According to the authors, the decrease in biting rates and a possible increase in the length of the extrinsic incubation period of the vector may cause the decrease in the incidence of dengue virus infections in man during this part of the year.

Estimates of Ae. aegypti population density employing human biting and landing counts, sweep net, aspiration-vacuum sweep of resting adults, and dipping collections for larvae and pupae are biased by a number of individual human traits dependent on the collectors (106). Other collecting techniques, developed to avoid these problems include the pyrethrin knockdown, oviposition traps and the one larvae per container techniques. The former test is biased by space, timing, and sealing problems, and a reluctance of the collectors to work in the presence of pyrethrin aerosol spray. The latter two techniques are better tools, but, they cannot be employed alone for population density estimates.

METHODS:

Study area: The study area for this investigation was a section of the Din Daeng area of Bangkok. The area is circumscribed by Prachasongkro Road on the north, Soi Charasongkhro on the west, Din Daeng I Road on the east and Klong Sam Sen on the south. Included in the area are 20 four-floor apartment buildings, many two-three story shop-houses, approximately 4 acres of confluent single story slum dwellings and a few single or two storey residential homes. The total human population of the study area was between 13,000 to 14,000.

Census of the population and mapping of the area: One hundred families of the Din Daeng study area that had a child attending Phibounprachasan School were randomly selected for the study. Each family was interviewed regarding address, income level, occupation, family size, ages, general health, and cultural and behavioral practices possibly related to disease recognition. Interviews of the families and the mosquito habitat surveys were conducted concurrently and on a seasonal basis. The entire area was mapped in regard to layout of housing, streets and other permanent landmarks.

Dengue virus infection of the human population: The seasonal incidence of dengue and Chikungunya infections was based on seroconversion rates determined by hemagglutination inhibition tests (107). Blood was obtained from family members before and after each season of the year. Overt dengue virus infections were determined by bi-weekly visits to the residence of each family in the study population. Classification of apparent and inapparent infection was based on previously defined criteria (108, 109).

Immature mosquito habitat surveillance: The availability and utilization of Ae. aegypti immature habitats were determined four times (each season of the year) between April 78-February 79. In March 79 the frequency of surveys for immature habitats was increased to every six weeks to gain more information for interpreting aegypti population trends. During the first two surveys in 1978, very little natural vegetation and no natural containers were found in the vicinity of the study residences. Accordingly, the immature habitat surveys concentrated on artificial containers. Residences of 100 families were surveyed both indoors and outdoors to provide an estimate of the number of potential and positive aegypti larval habitats. Families leaving the present study for any of a variety of reasons were replaced with randomly selected families in the same study area. All artificial containers within the boundary of each family residence were surveyed. Plastic containers, pails, washpans and other containers emptied on a daily basis were recorded, but not included since they did not usually provide a source for immature mosquitoes and therefore rarely contributed to the aegypti population.

Artificial containers defined as "inside" were determined on the basis of a roof or roof-like structure that precluded rain water from entering the container. Containers defined as "outside" were

determined on the basis of no roof or roof-like structure, or if a roof was present, it did not preclude rain water from entering the container. Lids or covers may occur on containers in both categories. Each artificial container with water was searched thoroughly for pupae and 1st to 4th stage larvae using a flashlight and a four ounce suction syringe. Water from containers that could not be visually searched, e.g., vases, was poured into a white pan, immature mosquitoes removed, and water then replaced in the original container. All immature mosquitoes were brought to the Laboratory, identified, separated on the basis of larvae or pupae, and then, either pooled for virus isolation or discarded.

Standardized water containers (ong jar) were purchased and one was placed in the residence of each of the 100 families selected for study. These jars will serve to provide estimates of the population density of Ae. aegypti obtained through concurrent employment of different, complementary sampling techniques. Each sampling device is being designed to focus on a certain life stage, behavioral factor, and/or physiological state of the mosquito population. The sampling methods (traps) described below are being designed to eliminate the bias of the human traits listed above and will be employed, provided preliminary tests show them to be reliable and effective sampling devices.

A floating larval trap will be used to determine the population density of Ae. aegypti larvae and pupae. These traps are used on a rotating schedule throughout the 100 family units and collections are made during one 24 hour period each week.

Emergence cone traps that fit over the mouth of water jars will be used to determine adult emergence patterns and densities during the year.

An estimate of the oviposition rate of Ae. aegypti will be determined seasonally by estimating the number of gravid females. This will be determined by capturing gravid females in a specially designed trap that captures the female, but does not allow her to reach a substrate for oviposition.

Additional sampling for estimating the adult density will be attempted by using a suction type trap. This trap will be designed to sample active flying adults, or by attracting them to a resting site.

RESULTS: Several aspects of Ae. aegypti biology and behavior, and their relationships to dengue virus transmission were reported previously (23). These studies involved: (A) the extrinsic incubation periods of dengue viruses 104 in aegypti; (B) transovarial transmission of dengue viruses by aegypti; and (C) the developmental periods and longevity of immatures and adults of aegypti under different conditions. Two of these studies have expanded during this study period. Consequently, they have been treated as separate studies and are reported elsewhere in this report, i.e., transovarial transmission and developmental and longevity studies. Due to technical problems there was little progress with the extrinsic incubation studies.

Vector competence studies. Experimental studies designed to determine the susceptibility of wild aegypti to infection with dengue viruses during the different seasons of the year, were discontinued due to the lack of a reliable method for infecting and demonstrating virus transmission by mosquitoes. In addition, studies could not be conducted to determine the susceptibility of selected strains of aegypti to dengue virus infection. The latter strains have been characterized according to their isoenzyme profiles as reported elsewhere in this report. A hanging drop technique was employed as described previously (23), and is somewhat similar to the method previously reported (110) to be effective for infecting Ae. aegypti with dengue viruses and for demonstrating virus transmission.

Although it was possible to infect low numbers of Ae. aegypti by allowing them to feed on a hanging drop of a dengue virus-blood suspension, the feeding rate was usually less than 10 percent. Of the mosquitoes that became infected, none transmitted detectable levels of virus to a hanging drop of blood after an incubation period of up to 35 days. Attempts to employ the hanging drop technique by others have resulted in limited success (111). These studies will be continued provided an effective technique can be developed. Meanwhile, eggs of aegypti, from the different seasons of the year will be stored, as well as eggs of the different colony strains of aegypti, to provide mosquitoes for testing. More recent findings (D.M. Watts, personal communication) indicate that rhesus monkeys infected with dengue viruses may prove to be a suitable host for infecting aegypti.

Adult and Larval Traps. The development of various larval and adult traps continued during this period and resulted in one larval trap and three adult traps which consistently yielded many adults

or larvae in laboratory tests. An emergence trap consisting of a clear plastic cone (35 cm tall) painted black inside, a funnel and a small clear plastic container, was designed to fit snugly over the top of the standardized (28 liter) water jars placed in each study residence. The plastic cone has a 3-4 cm hole at the tip which fits inside a funnel with a 4 cm long spout. The funnel spout is then inserted through a hole drilled through the bottom of a clear circular 13 cm wide and 8 cm deep plastic container (with lid) that traps the mosquitoes. This trap was placed over the study jars containing 4th stage aegypti larvae for three time periods in laboratory tests, 24, 48 and 72 hours. During laboratory tests, the traps set for 48 hours consistently yielded a large percentage of the hatching mosquitoes and kept a majority of the adults alive. The emergence trap became part of the routine field surveillance program in the Din Daeng Study area in January 1978 (see Field Studies).

Another adult trap was designed to simulate an oviposition site and trap the females that entered. This trap consisted of a large, round clear plastic container (16.5 cm diam. x 26.5 cm tall) painted black on the outside, with a large hole cut in the lid into which a funnel fits snugly (top of funnel flush with the lid) and with the spout pointing downward inside the container. Inside this container a small clear plastic container (trap cage) is attached to the funnel spout by inserting the spout through a small hole in the container lid. The bottom of the small inside container was at least 5-7 cm above the bottom of the large container so that about 4 cm of water (from aegypti positive water jars) could be placed in the large container. In addition, the funnel had several small screened panels in the cone portion so that water vapor from inside the trap could be detected in the funnel. Female aegypti attracted to the combination of black container-water vapor would fly down into the trap via the funnel spout and become trapped in the small container below, because they could not find the small funnel spout entrance again.

In laboratory tests with known numbers of mosquitoes, this trap worked very well, and was found equally effective for both sexes, gravid females and non-gravid females of Ae. aegypti and Culex quinquefasciatus. Based on the laboratory tests the trap was presumed to be generally attractive as a resting site, and became part of the field studies in Din Daeng in April 1978. After a one month period in the field study residences, however, this trap did not capture a sufficient number of specimens to warrant its continued use. Probable reasons for this failure outside the

laboratory are: (A) low adult aegypti numbers in the study residences (see emergence trap data); (B) competition with numerous other artificial water containers in the study residences (see the container indices for each type of residence); and (C) the failure of the black jar to be attractive in the poorly lit, dark interiors of most of the study residences.

The third trap consisted of a motorized suction trap (CDC) run on four flashlight batteries. The light source and lid of this standard trap were removed and replaced with a glossy black, square wooden box (8 cm tall x 19 cm on each side) with a hole in the bottom, which fit snugly over the top of the plastic frame of the CDC trap. Along each of the four sides of the black box there was a single narrow (10-15 mm) air vent through which air was drawn by the trap motor. The trap was designed so that adult aegypti would be attracted to the shiny black box and sucked through the lateral vents down into the standard net bag below the trap. In consecutive 24 hour (1200 to 1200) laboratory tests this trap consistently captured large numbers of adults, particularly male aegypti. After many laboratory trials, this trap was integrated into the field studies in Din Daeng in April 1979. However, as with the above resting trap, the number of adults captured during a nine month field trial was too small to warrant its continued use. Beside low adult aegypti numbers in the study residences and the failure of the black box to attract adults in the dimly lit interiors, other problems were encountered. The large size of the trap in the very small (one room) residences caused some discomfort to the residents. In addition, finding a means to suspend the trap caused difficulties in some residences. This trap uniformly caught a fair number of Culex quinquefasciatus in the study residences; however, a standard CDC trap with light, probably would have caught many more adults of this latter species.

During the last reporting period a very efficient floating larval trap was designed and tested in the laboratory (23). This trap will float in any water container holding 10 cm or more water and is ideally suited for trapping in the ceramic water jars, cement bath tanks and metal drums which are used to store water in the tropics. The trap keeps the larvae/pupae alive during a 24 hour trapping period, is very stable, quickly returning to an upright position if turned over, and floats randomly on the water surface, eliminating the human bias of collecting only in concentrations of larvae.

During this reporting period laboratory tests with the larval trap were concluded and this trap was integrated into the Ding

Daeng field studies in January 1979 (see Field Studies).

FIELD STUDIES: Dengue virus infection of the human population. The prevalence of antibody to dengue virus in those families resident in high-rise apartments (flats) differed from those families living in shop houses or the slums. There was no observed difference in the last two and they have been combined for purposes of this report. Figures 6 and 7 present the 1978 pre-dengue season prevalence of antibody against dengue viruses and Chikungunya virus, respectively. While the prevalence of anti-Chikungunya antibody varied only slightly by dwelling type, housing appeared to have a noticeable effect on dengue antibody in children. Regardless of housing, dengue antibody prevalence approached 100 percent by age 20.

Figures 8 and 9 present the incidence of dengue virus infection in Din Daeng residents. Figure 8 represents only those individuals who did not have evidence of antibody prior to the start of the period under examination. Twenty-six primary seroconversions were documented during the early rainy season (June-September 1978) and eight were observed during the late rainy-early cool season (September 1978-January 1979). The incidence between housing types was not statistically significant, but the difference in antibody acquisition between the two periods was noticeable ($\chi^2 = 5.98$, $p < 0.02$). Figure 9 presents the data for the same periods in individuals who had previously exhibited an antibody titer to one or more dengue types. In contrast to primary infections, the secondary type response was not different between the two periods. Shop and slum housing accounted for significantly more secondary infections in the late rainy season than did the flats ($\chi^2 = 15.96$, $p < 0.005$). This was the only subgroup in which housing type was associated with a difference in the incidence of dengue infection.

Artificial container surveillance. Investigations of the seasonal availability and utilization of artificial containers by Ae. aegypti in the Din Daeng study area were initiated in April 1978 (23) and have continued to the present. The percentage and frequency of potential artificial containers with water are shown in Figure 10. The percentage of containers with water inside residences ranged from 69.5 to 84.6 percent, while the containers with water outside residences ranged from 94.6 to 100.00 percent, except for the initial survey when it was 61.2 percent. However, during each survey the frequency of inside containers with water was six times greater than the frequency of outside containers with water. The low number of outside artificial containers with

water at the study residences was probably due to: (A) the limited space at most residences, leaving little room for more than a dwelling; and (B) the structural design of the high-rise flats. Outside containers were mostly confined to the slum residences, with a few located in the shop residences. High-rise flats (approximately 50% of the total residences surveyed) because of vertical design, did not have outside space for outside containers.

The frequency and percentage of artificial containers positive for aegypti are shown in Figure 11. The percentage of artificial containers positive for aegypti was higher outside than inside residences during all trapping periods. However, since slum residences contribute the majority of outside containers, this phenomenon appears to be based on residence-type rather than outside versus inside containers.

Although inconclusive at this time, the data indicate that the mosquito population density, in terms of number of positive containers, is influenced by both mean monthly temperature and precipitation (Figure 12). It appears that changes in the percentage and frequency of artificial containers positive for Ae. aegypti are reflections of changes in the mean monthly temperatures, i.e., with increases in the mean monthly temperature there are observed increases in the number of positive artificial containers, and conversely, decreases in the mean monthly temperature result in lower numbers of positive artificial containers.

Increases in the percentage and frequency of positive containers are also associated with periods of increased precipitation, i.e., during the rainy season. It appears that the mosquito population density, as determined by frequency of positive artificial containers, is first influenced by increases in the mean monthly temperature, beginning with the hot dry season. With the end of the hot dry season and the beginning of the rainy season, the frequency of positive artificial containers continued to increase. However, near the end of the rainy season and the beginning of the cool dry season, a decrease in the frequency of positive containers was observed. The observed changes in percent of positive containers outside residences are more noticeable than for containers inside residences, whereas changes in frequency are much smaller. This difference is primarily due to the limited numbers of artificial containers outside the study residences.

Outside containers, with the exception of ong jars, are also dependent upon rain as a water source. However most of the outside containers were ong jars and were filled by rain and/or tap water.

The frequency and percentage of different types of containers positive for aegypti inside and outside study residences are summarized in Table 30. Three major types of artificial containers (ong jars, bath and foot basin, and ant-traps) were found to be the primary habitats for immature aegypti. A fourth type, flower vase/base, although relatively abundant, was positive infrequently (< 3.3 percent).

Containers classified as "others", although included, were not considered to be a major contributing factor primarily because of their small numbers and their very low positive rate. Slum residences are almost totally responsible for all "other" containers. Most slum residences have a small area adjacent to the house where small children play or water containers are stored, and occasional "other" containers are stored or discarded.

Comparing all of the containers, a higher percentage of ant traps were positive, ranging from 20.8 to 52.5 percent for all trapping periods. Ong jars and bath and foot basins were next, ranging from 13.1 to 39.1 and 9.8 to 32.5 percent respectively (Figure 13). Although bath and foot basins and ant traps, when present, were frequently observed to be positive, they were limited in number and in the case of bath and foot basin, were almost entirely restricted to high-rise flats and shop residences. For this reason, it became necessary to obtain a "positive container index" for the different types of residences, i.e., the number of positive containers per residence. This provides a more reliable index in evaluating seasonal population changes and an estimation of the type of containers most responsible for changes in the seasonal abundance of aegypti (Tables 31, 32, 33 and 34). Overall, ong jars are more frequently observed positive than all other containers combined (0.31 to 1.94). Ant traps and bath and foot basins follow, ranging from 0.12 to 0.33 and 0.05 to 0.13, respectively (Figure 14).

The high-rise flat residences, in general, have a very low positive container index when compared to the other type residences. The containers most frequently positive were ong jars, with bath and foot tanks, ant traps and flower vases/bases positive only occasionally (Figure 15). Of the three types of study residences, slum residences had the highest positive container index (Figure 16).

Ong jars accounted for the majority of positive containers, while the remainder of positive containers were primarily ant traps. Bath and foot tanks were infrequent in the slum residences and infrequently positive. Shop residences, unlike slum residences, do not have one type of container accounting for the majority of all positive containers (Figure 17'). Also shop residences were the only type residence where there was a significant number of positive flower vase/bases.

Changes in the mean number of different types of containers infested with Ae. aegypti were similar, in most cases, to the total seasonal fluctuation in positive inside and outside containers. Ong jars, because of their greater numbers and common use in all three residence types, appear to provide a better index of mosquito population density changes in relation to frequency of positive containers. However, a more in depth evaluation of mosquito population changes can be provided for shop residences, when ong jars, ant traps and bath and foot basins are included in a surveillance program.

Percent seasonal changes in the study residences having different types of containers positive for aegypti are summarized in Table 35. The percentage of all study residences positive (Figure 18) is directly associated with changes in the mean number of positive containers. The percentage of high-rise flat residences positive for aegypti varied from 4.2 to 26.5 percent for all surveys and was very small when compared with shop and slum residences. The percentage of shop and slum residences positive for aegypti were similar, varying from 45.0 to 88.2 and 51.6 to 84.4 percent respectively. Although the total percentage of positive shop and slum residences were nearly the same for each trapping period, the type of container which contributed to the residences being positive varied. Ong jars were almost totally responsible for slum residences being positive, with bath tanks accounting for less than seven percent and ant traps for less than 17 percent during any one trapping period. In general, if a slum residence had positive bath tanks and/or ant traps, it also had at least one positive ong jar. Although ong jars play a major role in shop residences being positive, they were responsible for more than 50 percent of shop residences being positive on only three occasions. Positive bath tanks were especially prevalent (15.0-52.6 percent) in shop houses, while ant traps and flower vases were positive between 20-35 percent of the time.

Figures 19, 20, 21 and 22 illustrate the uneven distribution of the different types of positive containers in the three types of residences. Slum houses were more frequently positive for ong jars than either shop or high-rise flat residences (Figure 19), while shop residences were more frequently positive for bath and foot tanks and flower vases (Figures 20 and 21.). There was no obvious differences in the number of positive ant traps between the slum and shop residences (Figure 22). Apparently, the primary difference between high-rise flats and shop residences was their respective accessibility to the mosquitoes. Both shops and flat residences had similar types and quantities of artificial containers. However, shops were open, and on ground level, allowing easy access for adult aegypti, while the four story high-rise flats usually had screened windows and occasionally screened doors, and had the entry way at least one story above ground level.

Mosquito population density surveillance. Originally, mosquito density estimates were going to be based on the results of four trapping programs, i.e., larval trap, emergence trap, black resting trap and the black box-suction trap. These traps were all developed and laboratory tested, with good to excellent results, by early 1979. However, as discussed in the laboratory studies section, only a few adult aegypti were collected by the black resting trap and the black box-suction trap after a one month test in the study residences. Accordingly, surveillance with these two traps was discontinued. The larval and emergence traps have been monitoring aegypti population densities since January 1979. Since routine container surveillance determines the prevalence of positive jars, the larval and emergence traps were set only in/on ong (water) jars that contained larvae or pupae. By this means data were accumulated on changes in the abundance of aegypti in known positive containers. The data for the larval and emergence traps are presented separately below.

Emergence trap surveillance. A uniform sized water container was selected to provide a standardized immature habitat for estimating the population densities of both emerging adults and immatures of aegypti, and to facilitate the use of uniform sized traps, particularly the larger emergence trap. Accordingly, a standardized ceramic water jar (AFRIMS ong jar: 40 cm x 30 cm, approximately 28 liter volume) was placed in each residence of the 100 families involved in the study. New randomly selected families replacing departing families were also provided with an AFRIMS jar. Broken or lost AFRIMS jars were replaced with new jars.

The schedule for setting the emergence traps each trapping period always preceded the larval trapping period. Using this schedule, the highly efficient larval traps did not have a detrimental effect on the adult aegypti emergence rates.

Four trapping periods have been completed and the results tabulated (Table 36). Emergence traps were set for approximately 48 hours, and only on aegypti positive AFRIMS jars. The actual trapping time ranged from 43.8 to 53.8 hours, with a mean of 47.9 hours. The prevalence of aegypti positive AFRIMS jars during any one survey was consistently low. Accordingly, only a few emergence traps were set each trapping period and the results are inconclusive. However, the data indicate a trend of increased emergence associated with rising mean monthly temperatures, which also continues to increase after the onset of the rainy season. Male: female ratios of adult aegypti captured in the emergence traps ranged from 1:0.7 to 1:1.4 for the different trapping periods. The average male:female ratio for the four trapping periods was 1:1. Data are currently insufficient for indicating seasonal male:female ratios.

Larval trap surveillance. Surveillance of immature mosquitoes by a larval trap was initiated January 1979, and five trapping periods have been completed. This trap was designed to provide an unbiased estimate of immature mosquito population densities, and to assess possible seasonal differences in aegypti abundance. Ong (water) jars, including AFRIMS ong jars, were trapped in all three categories of residences, i.e., high-rise flats, shops and slums. Ong jars were selected as the source of surveillance primarily because of: (A) a large numbers of ong jars in the study area; (B) previous surveys demonstrated a large percentage of the jars were positive; (C) their abundance in all of the types of study residences surveyed; and (D) physical limiting factors of the trap design, primarily its size (13 cm diam. x 13 cm tall). All ong jars with water were surveyed in the study residences. Larval traps were set only in ong jars with observed immature mosquitoes, and removed approximately 24 hours later.

The actual range and mean of total time for completed larval trap surveys were 17.9 to 28.8 hours and 23.6 hours, respectively. Immature mosquitoes captured in the larval traps were separated as pupae or larvae, identified and recorded. Larval trap surveillance will continue into 1980.

The positive jar index (Figure 23) more accurately reflects the actual aegypti positive jar rate per type of residence than the

percentage of positive ong jars per type of residence. This is due to a disparity in the average number of ong jars per residence, per type of residence (high-rise flat 2.3, shop 2.7, slum 4.9). The tabulated data also indicate that the percentage of ong jars positive for aegypti increases following the onset of increased amounts of precipitation (Figures 12 and 24). Although all three types of residences demonstrated an increase in positive ong jars following the onset of periods of increased precipitation the slum residences had the greatest increase, while the high-rise flat residences had the smallest.

The average number of larvae, pupae and combined total¹ of immature mosquitoes per sampled² ong jar for the study residences are summarized in Table 37. More trapping is required to get a better perspective of the cyclic nature of immature aegypti populations in relation to mean monthly temperature and precipitation. However, the data accrued to date, indicate that the average number of mosquitoes per sampled ong jar increases with the beginning of the hot season and even further after the onset of rainy periods. The frequency of immature aegypti trapped per sampled ong jar ranged from 0 to 512, with less than five percent of the jars negative. The high-rise flats had the highest average number of immature aegypti per positive ong jar (positive ong jar index), while the slums had the lowest number. However, the reverse is true when comparing the total number of ong jars with water, i.e., the slums had the highest average numbers of immature aegypti per ong jar (ong jar index) while the high-rise flats had the lowest. The relationships of mean monthly temperature and precipitation to the number of pupae as compared to the numbers of larvae are currently inconclusive.

Similar mosquito density-meteorological relationships although less conclusive, were also demonstrated for the seasonal frequency and percent of different types of study residences with ong jars infested with immature aegypti. The slums demonstrated the highest percentage of positive residences (76.5-83.3 percent), while the high-rise flats had the lowest percentage positive residences (4.2-16.7 percent). The shop residences were intermediate and ranged from 41.2-50.0 percent positive for all five trapping periods. The

¹ Larvae and pupae not separated for first two trapping phases.

² Occasionally we were not allowed trapping access to positive jars.

TABLE 30 Frequency and percentage of inside and outside containers¹ with water positive for immature Aedes aegypti in study residences by surveys, Din Daeng, Bangkok, Thailand 1978-79.

CONTAINERS	DATE OF SURVEY									
	3-18 APR 78	30 MAY - 15 JUN 78	18-28 SEPT 78	6-15 DEC 78	5-22 MAR 79	16 APR - 4 MAY 79	29 MAY - 15 JUN 79	9-27 JUL 79		
Water jar	13.1 (31/236)	22.7 (57/251)	25.0 (77/308)	18.2 (54/297)	29.1 (97/333)	26.2 (83/317)	31.8 (101/318)	39.1 (124/317)		
Bath & Foot tanks	9.8 (5/51)	32.5 (13/40)	27.7 (13/47)	14.0 (7/50)	8.9 (4/45)	10.6 (5/47)	23.4 (11/47)	22.9 (11/48)		
Flower vase & clay pot base	2.7 (4/149)	3.3 (4/120)	1.4 (2/146)	2.1 (3/140)	2.4 (4/164)	2.2 (3/134)	0.64 (1/155)	1.9 (3/155)		
Ant trap	28.2 (22/78)	52.4 (33/63)	20.8 (16/77)	26.1 (12/46)	34.7 (17/49)	38.1 (16/42)	50.0 (18/36)	48.7 (19/39)		
Other	26.7 (4/15)	9.1 (3/33)	0.0 (0/7)	33.3 (2/6)	50.0 (2/4)	100.0 (1/1)	25.0 (2/8)	66.7 (2/3)		
TOTAL	12.5 (66/529)	22.5 (110/489)	18.5 (108/585)	14.5 (78/539)	20.8 (124/595)	20.0 (108/541)	23.6 (133/564)	28.3 (159/562)		

¹ Plastic containers, pails, wash pans and other containers emptied on a daily basis not included.

TABLE 31 Positive container index (mean) for immature Aedes aegypti per study residence (outside & inside), Din Daeng, Bangkok, Thailand, 1978-79.

CONTAINERS	DATE OF SURVEY							
	3-18 APR 78	30 MAY - 15 JUN 78	18-28 SEPT 78	6-15 DEC 78	5-22 MAR 79	16 APR - 4 MAY 79	29 MAY - 15 JUN 79	9-27 JUL 79
Water Jar	0.31 (31/100)	0.57 (57/100)	0.77 (77/100)	0.55 (54/99)	0.98 (97/99)	0.86 (83/97)	1.05 (101/96)	1.94 (124/94)
Bath & Foot Tanks	0.05 (5/100)	0.13 (13/100)	0.13 (13/100)	0.07 (7/99)	0.04 (4/99)	0.05 (5/97)	0.11 (11/96)	0.12 (11/94)
Flower Vase & Clay Pot Base	0.04 (4/100)	0.04 (4/100)	0.02 (2/100)	0.03 (3/99)	0.04 (4/99)	0.03 (3/97)	0.01 (1/96)	0.03 (3/94)
Ant Trap	0.22 (22/100)	0.33 (33/100)	0.16 (16/100)	0.12 (12/99)	0.17 (17/99)	0.16 (16/97)	0.19 (18/96)	0.20 (19/94)
Other ¹	0.04 (4/100)	0.03 (3/100)	0.0 (0/57)	0.02 (2/99)	0.02 (2/99)	0.01 (1/97)	0.02 (2/96)	0.02 (2/94)
TOTAL	0.66 (66/100)	1.10 (110/100)	1.08 (108/100)	0.79 (78/99)	1.18 (117/99)	1.11 (108/97)	1.39 (133/96)	1.69 (159/94)

¹ Plastic containers, pails, wash pans and other artificial containers emptied on a daily basis not included.

TABLE 32 Positive container index for immature Aedes aegypti per high-rise flat (inside) Din Daeng, Bangkok, Thailand (1978-79).

CONTAINERS	DATE OF SURVEY									
	3-18 APR 78	30 MAY - 15 JUN 78	18-28 SEPT 78	6-15 DEC 78	5-22 MAR 79	16 APR - 4 MAY 79	29 MAY - 15 JUN 79	9-27 JUL 79		
Water Jar	0.10 (5/49)	0.16 (8/49)	0.27 (13/49)	0.16 (8/49)	0.10 (5/48)	0.15 (7/48)	0.17 (8/47)	0.25 (12/48)		
Bath & Foot Tanks	0.02 (1/49)	0.02 (1/49)	0.06 (3/49)	0.02 (1/49)	0.02 (1/48)	0.0 (0/48)	0.0 (0/47)	0.08 (4/48)		
Flower Vase Clay Pot Base	0.0 (0/49)	0.06 (3/49)	0.0 (0/49)	0.0 (0/49)	0.04 (2/48)	0.0 (0/48)	0.0 (0/47)	0.0 (0/48)		
Ant Trap	0.14 (7/49)	0.06 (3/49)	0.04 (2/49)	0.0 (0/49)	0.0 (0/48)	0.0 (0/48)	0.09 (4/47)	0.04 (2/48)		
Other ¹	0.0 (0/49)	0.0 (0/49)	0.0 (0/49)	0.0 (0/49)	0.0 (0/48)	0.0 (0/48)	0.0 (0/47)	0.0 (0/48)		
TOTAL	0.27 (13/49)	0.30 (15/49)	0.37 (18/49)	0.18 (9/49)	0.17 (8/48)	0.15 (7/48)	0.26 (12/47)	0.38 (18/48)		

¹ Plastic containers, pails, wash pans and other artificial containers emptied on a daily basis not included.

TABLE 33 Positive container index for immature Aedes aegypti per shop residence (outside-inside)
Din Daeng, Bangkok, Thailand(1978-79).

CONTAINERS	DATE OF SURVEY							
	3-18 APR 78	30 MAY - 15 JUN 78	18-28 SEPT 78	6-15 DEC 78	5-22 MAR 79	16 APR - 4 MAY 79	29 MAY - 15 JUN 79	9-27 JUL 79
Water Jar	0.15 (3/20)	0.32 (6/19)	0.47 (8/17)	0.35 (6/17)	0.82 (14/17)	0.69 (11/16)	0.94 (16/17)	1.13 (18/16)
Bath & Foot Tanks	0.15 (3/20)	0.5 (10/19)	0.53 (9/17)	0.35 (6/17)	0.18 (3/17)	0.31 (5/16)	0.59 (10/17)	0.38 (6/16)
Flower Vase Clay Pot Base	0.2 (4/20)	0.05 (1/19)	0.12 (2/17)	0.18 (3/17)	0.11 (2/17)	0.19 (3/16)	0.06 (1/17)	0.19 (3/16)
Ant Trap	0.2 (4/20)	0.79 (15/19)	0.29 (5/17)	0.18 (3/17)	0.35 (6/17)	0.44 (7/16)	0.24 (4/17)	0.38 (6/16)
Other ¹	0.0 (0/20)	0.0 (0/19)	0.0 (0/17)	0.0 (0/17)	0.0 (0/17)	0.0 (0/16)	0.0 (0/17)	0.0 (0/17)
TOTAL	0.7 (14/20)	1.7 (32/19)	1.41 (24/17)	1.06 (18/17)	1.47 (25/17)	1.63 (26/16)	1.82 (31/17)	2.06 (33/16)

¹ Plastic containers, pails, wash pans and other artificial containers emptied on a daily basis not included.

TABLE 34 Positive container index for immature Aedes aegypti per slum residence (outside-inside)
Din Daeng, Bangkok, Thailand (1978-79)

CONTAINERS	DATE OF SURVEY									
	3-18 APR 78	30 MAY - 15 JUN 78	18-28 SEPT 78	6-15 DEC 78	5-22 MAR 79	16 APR - 4 MAY 79	29 MAY - 15 JUN 79	9-27 JUL 79		
Water Jar	0.74 (23/31)	1.34 (43/32)	1.65 (56/34)	1.21 (40/33)	2.09 (71/34)	1.97 (65/33)	2.41 (77/32)	3.13 (94/30)		
Bath & Foot Tanks	0.03 (1/31)	0.06 (2/32)	0.03 (1/34)	0.0 (0/33)	0.0 (0/34)	0.0 (0/33)	0.03 (1/32)	0.03 (1/30)		
Flower Vase Clay Pot Base	0.0 (0/31)	0.0 (0/32)	0.0 (0/34)	0.0 (0/33)	0.0 (0/34)	0.0 (0/33)	0.0 (0/32)	0.0 (0/30)		
Ant Trap	0.35 (11/31)	0.47 (15/32)	0.26 (9/34)	0.27 (9/33)	0.32 (11/34)	0.27 (9/33)	0.31 (10/32)	0.37 (11/30)		
Other ¹	0.13 (4/31)	0.09 (3/32)	0.0 (0/34)	0.06 (2/33)	0.06 (2/34)	0.03 (1/33)	0.06 (2/32)	0.07 (2/30)		
TOTAL	1.26 (39/31)	1.97 (63/32)	1.94 (66/34)	1.55 (51/33)	2.47 (84/34)	2.27 (75/33)	2.81 (90/32)	3.60 (108/30)		

¹ Plastic containers, pails, wash pans and other artificial containers emptied on a daily basis not included.

TABLE 35 Percent seasonal changes in study residences having different types of containers positive for *Aedes aegypti*, Din Daeng, Bangkok, Thailand, 1978-79.

CONTAINERS	DATE OF SURVEY							
	3-18 APR 78	30 MAY - 15 JUN 78	18-28 SEPT 78	6-15 DEC 78	5-22 MAR 79	16 APR - 4 MAY 79	29 MAY - 15 JUN 79	9-27 JUL 79
Water Jar	19.0 (19/100)	29.0 (29/100)	39.0 (39/100)	30.3 (30/99)	38.4 (38/99)	36.1 (35/97)	37.5 (36/96)	45.7 (43/94)
Bath & Foot Tanks	5.0 (5/100)	13.0 (13/100)	12.0 (12/100)	7.1 (7/99)	4.0 (4/99)	4.1 (4/97)	9.4 (9/96)	10.6 (10/94)
Flower Vase & Clay Pot Base	2.0 (2/100)	3.0 (3/100)	2.0 (2/100)	2.0 (2/99)	4.0 (4/99)	2.1 (2/97)	0.0 (0/96)	1.1 (1/94)
Ant Trap	10.0 (10/100)	9.0 (9/100)	8.0 (8/100)	5.1 (5/99)	5.1 (5/99)	7.2 (7/97)	6.3 (6/96)	8.5 (8/94)
Other ¹	3.0 (3/100)	3.0 (3/100)	0.0 (0/100)	1.0 (1/99)	2.0 (2/99)	1.0 (1/97)	2.1 (2/96)	2.1 (2/96)
TOTAL ²	29.0 (29/100)	43.0 (43/100)	53.0 (53/100)	38.4 (38/99)	42.4 (42/99)	42.3 (41/97)	45.8 (44/96)	53.2 (50/94)
INSIDE CONTAINERS	25.0 (25/100)	37.0 (37/100)	46.0 (46/100)	34.3 (34/99)	36.4 (36/99)	33.0 (32/97)	42.7 (41/96)	51.1 (48/94)
OUTSIDE CONTAINERS	8.0 (8/100)	14.0 (14/100)	15.0 (15/100)	10.1 (10/99)	17.2 (17/99)	17.5 (17/97)	13.5 (13/96)	16.0 (15/94)

¹ Plastic containers, pails, wash pans and other artificial containers emptied on a daily basis not included.

² Residences positive for more than one type of container or positive both inside and outside only counted once.

Table 36 Emergence Trap: Seasonal variation of positive emergence traps for AFRIMS ong jar¹ for houses, Din Daeng, Bangkok, Thailand, 1979.

Date of Survey	Number Jars Surveyed		Number Jars Positive for <u>A. aegypti</u> Larvae & Pupae		Number of Emergence Traps Positive		Percent Traps Positive for Positive Jars	
	Inside	Outside Total	Inside	Outside Total	Inside	Outside Total	Inside	Outside Total
22 JAN - 15 FEB 79	75	6 81	24	5 29	9	5 14	37.5	100.0 48.3
5-21 MAR 79	76	7 83	17	3 20	10	1 11	58.8	33.3 55.0
16 April- 4 May 79	74	6 80	16	3 19	4	0 4	25.0	0.0 21.1
9-27 July 79	79	1 80	24	1 25	13	0 13	54.2	0.0 52.0

¹ One standard size ong jar (40 cm x 80 cm) was placed in each household for trapping purposes.

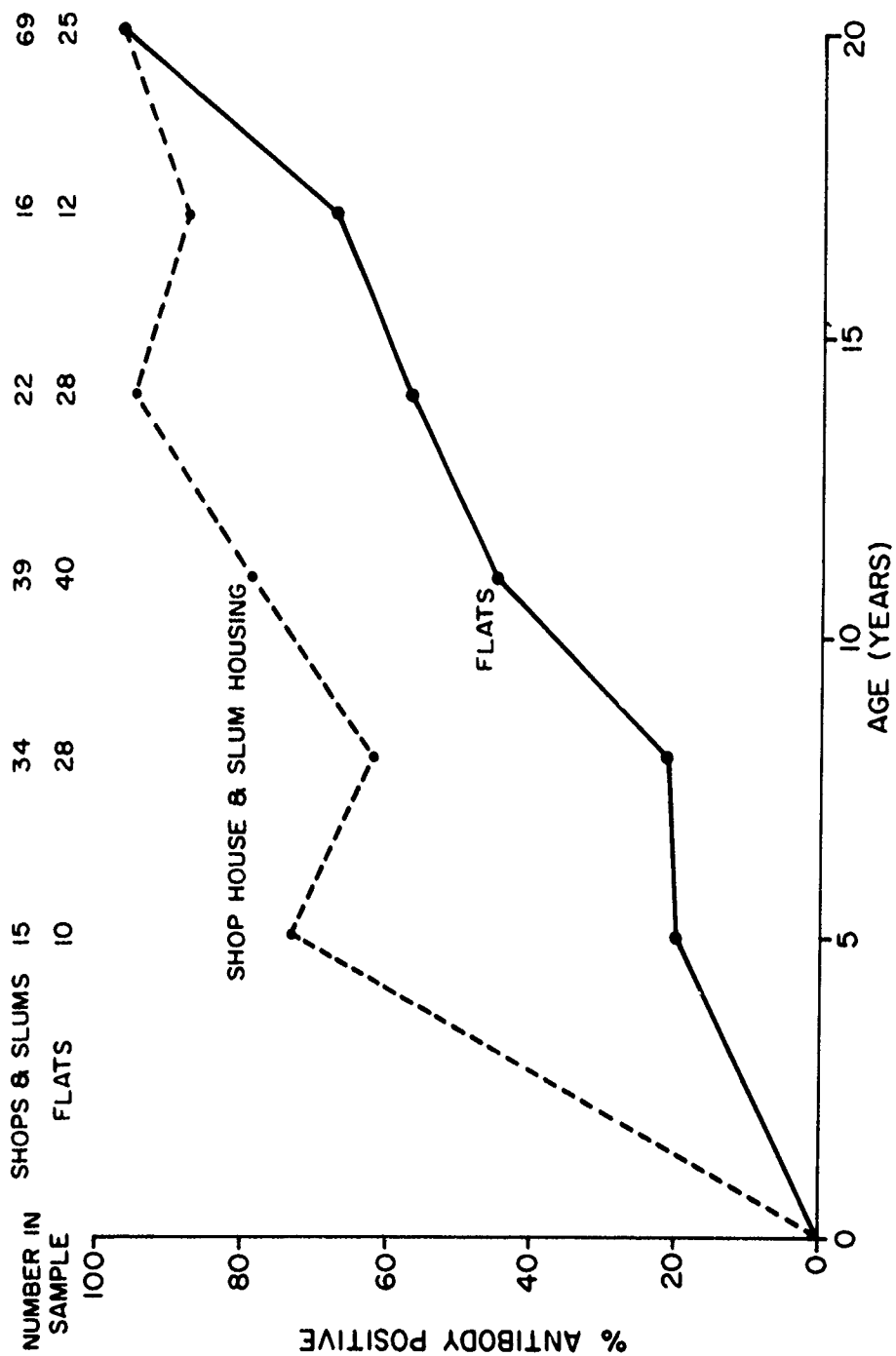


FIG. 6 PREVALENCE OF ONE OR MORE DENGUE ANTIBODY TITERS, DIN DAENG, THAILAND 1978

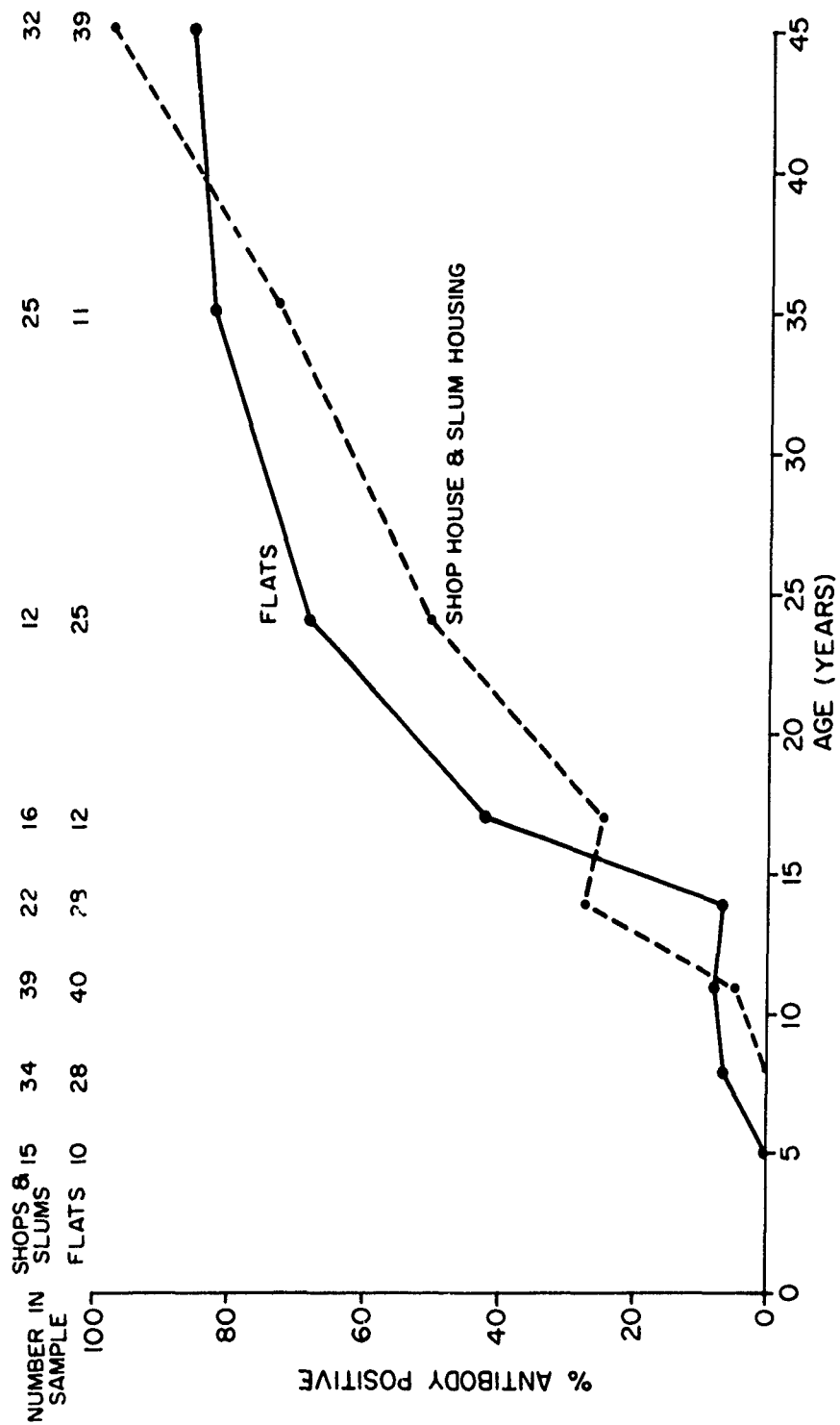


FIG. 7. PREVALENCE OF CHIKUNGUNYA VIRUS ANTIBODY, DIN DAENG, THAILAND 1978.

FIG. 8 INCIDENCE OF DENGUE ANTIBODY ACQUISITION IN PREVIOUSLY UNINFECTED INDIVIDUALS
DIN DAENG, BANGKOK THAILAND.

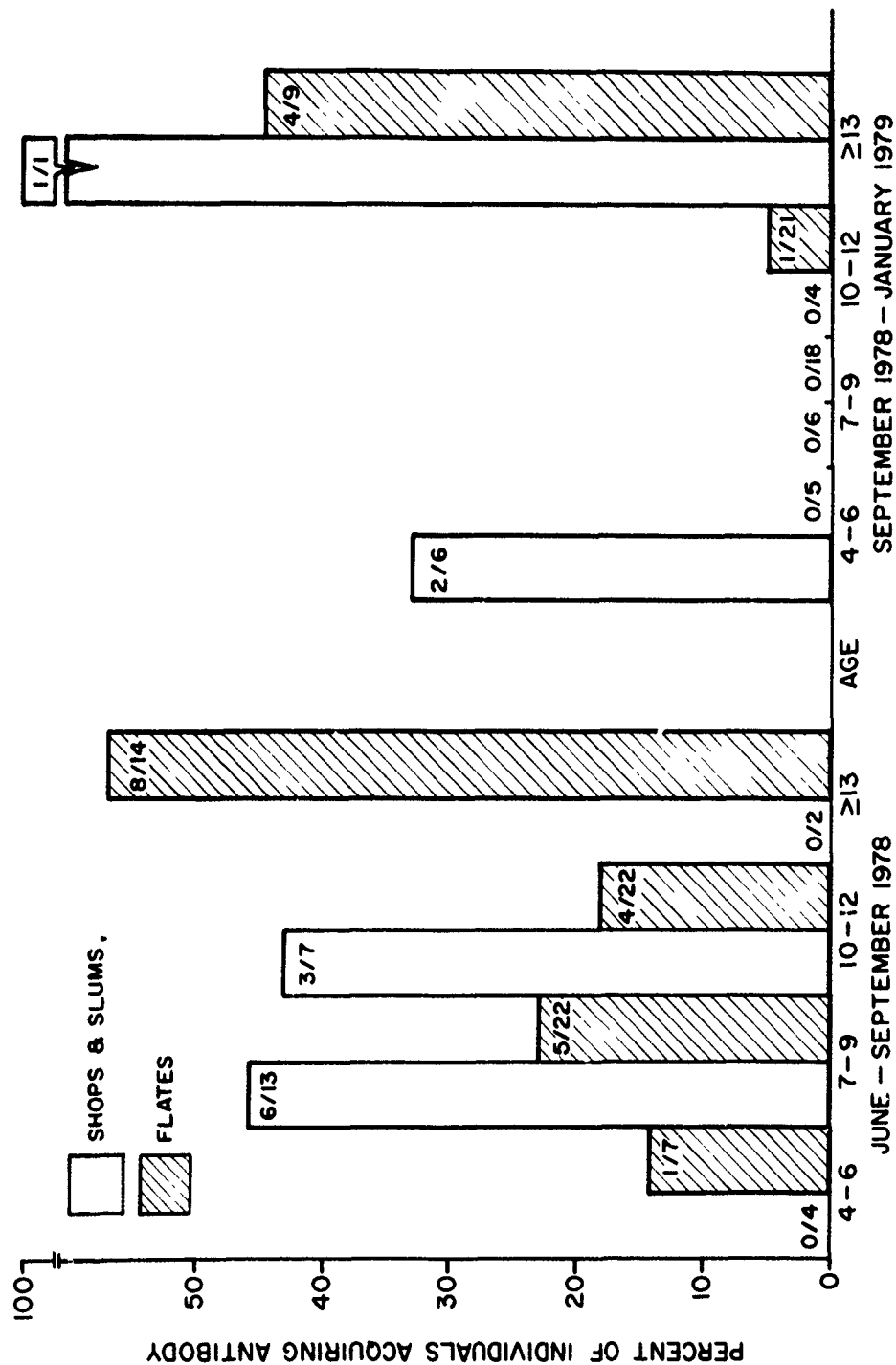
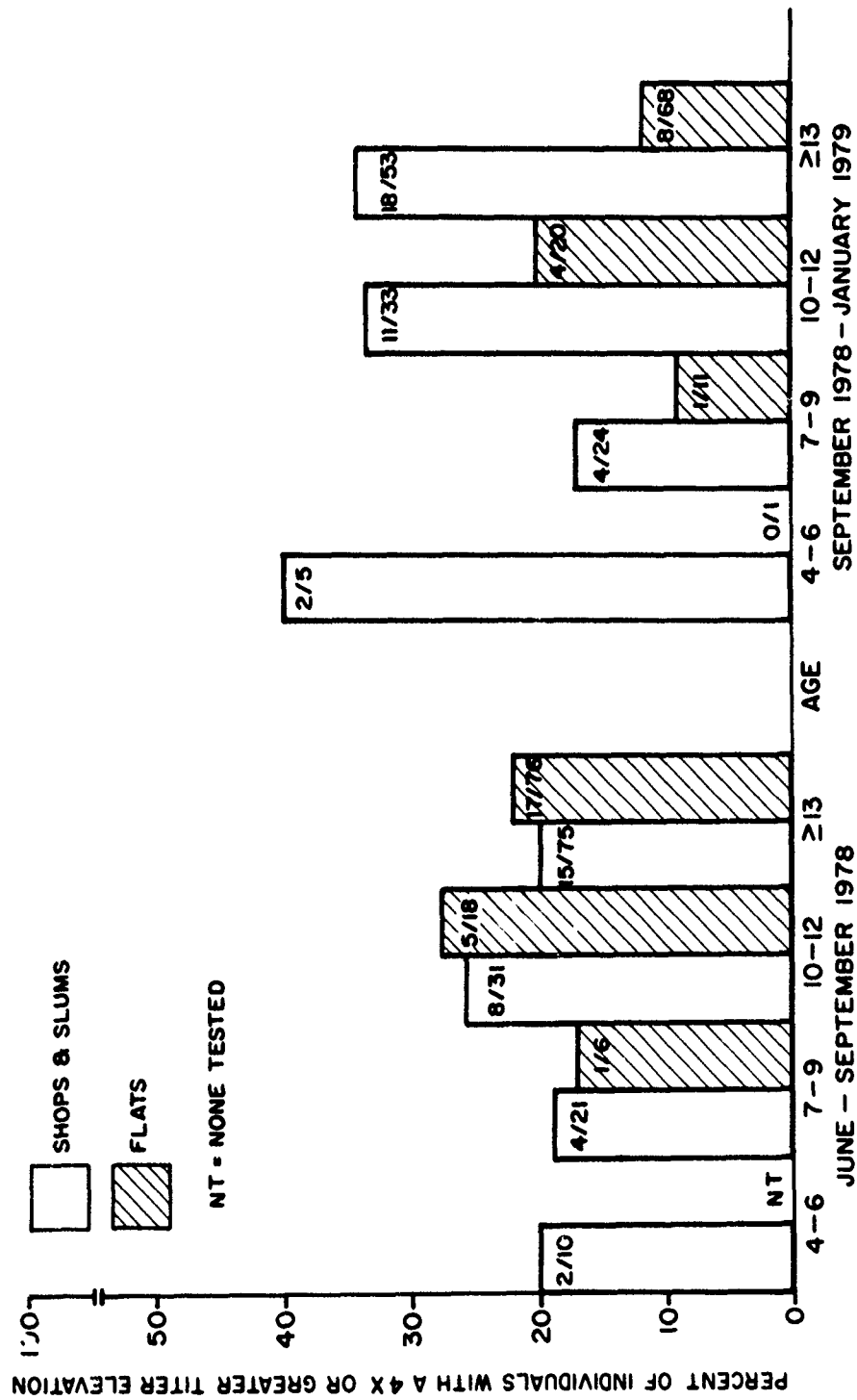


FIG. 9 INCIDENCE OF DENGUE TITER ELEVATION IN PREVIOUSLY INFECTED INDIVIDUALS
DIN DAENG, BANGKOK THAILAND.



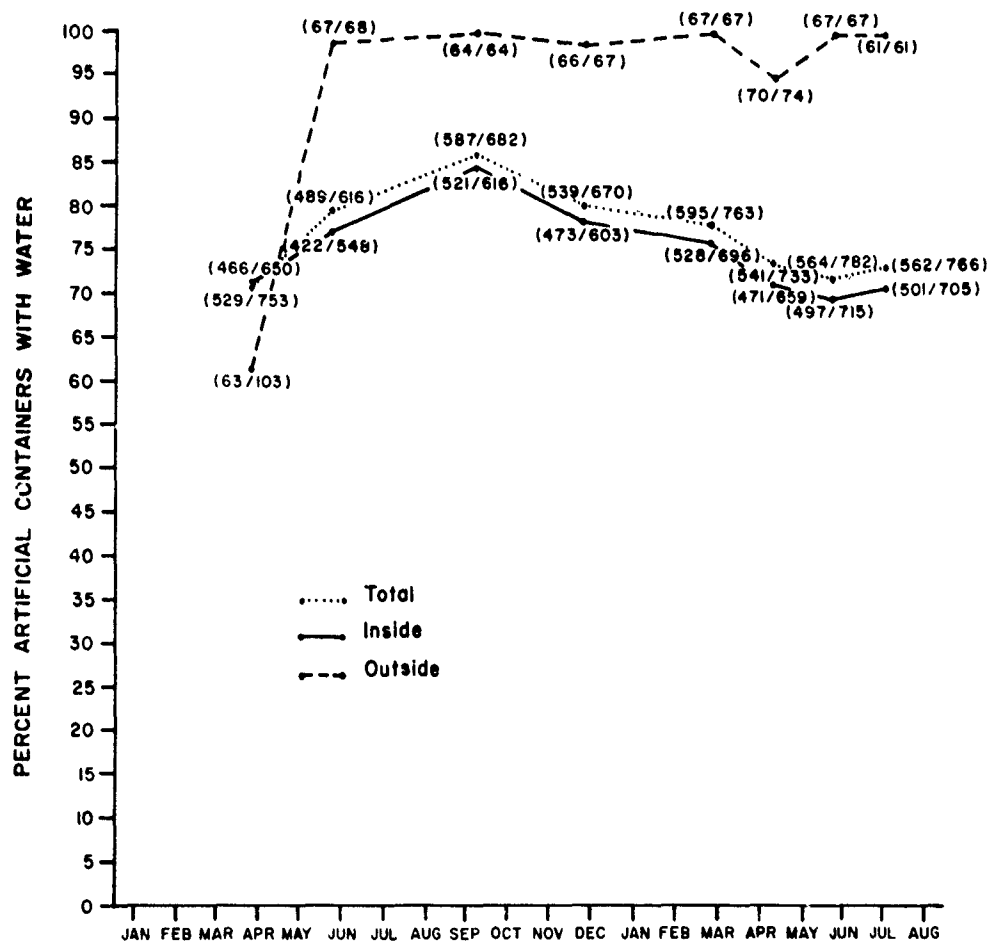


Figure 10 Percentage of artificial water containers¹ with water in study residences, Din Daeng, Bangkok, Thailand (1978-1979).

¹ Plastic containers, pails, wash pans and other artificial containers emptied on a daily basis not included.

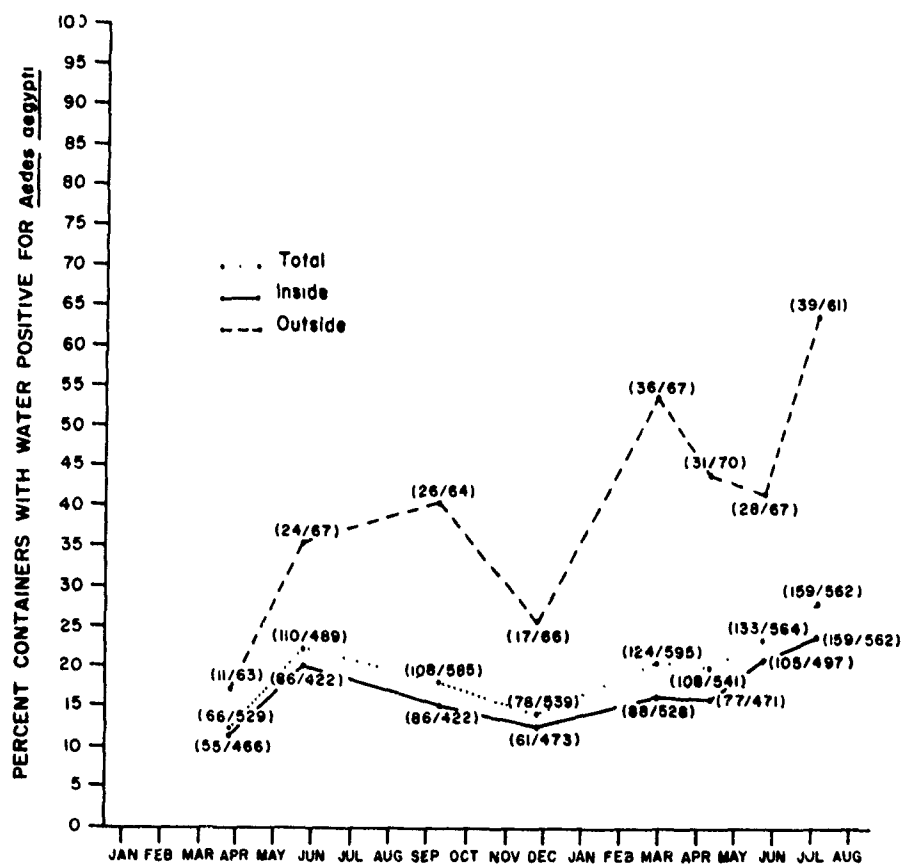


Figure 11 Percent artificial containers¹ with water positive for immature *Aedes aegypti* inside and outside residences by seasons, Din Daeng, Bangkok, Thailand (1978-1979).

¹ Plastic containers, pails, wash pans, and other artificial containers emptied on a daily basis not included.

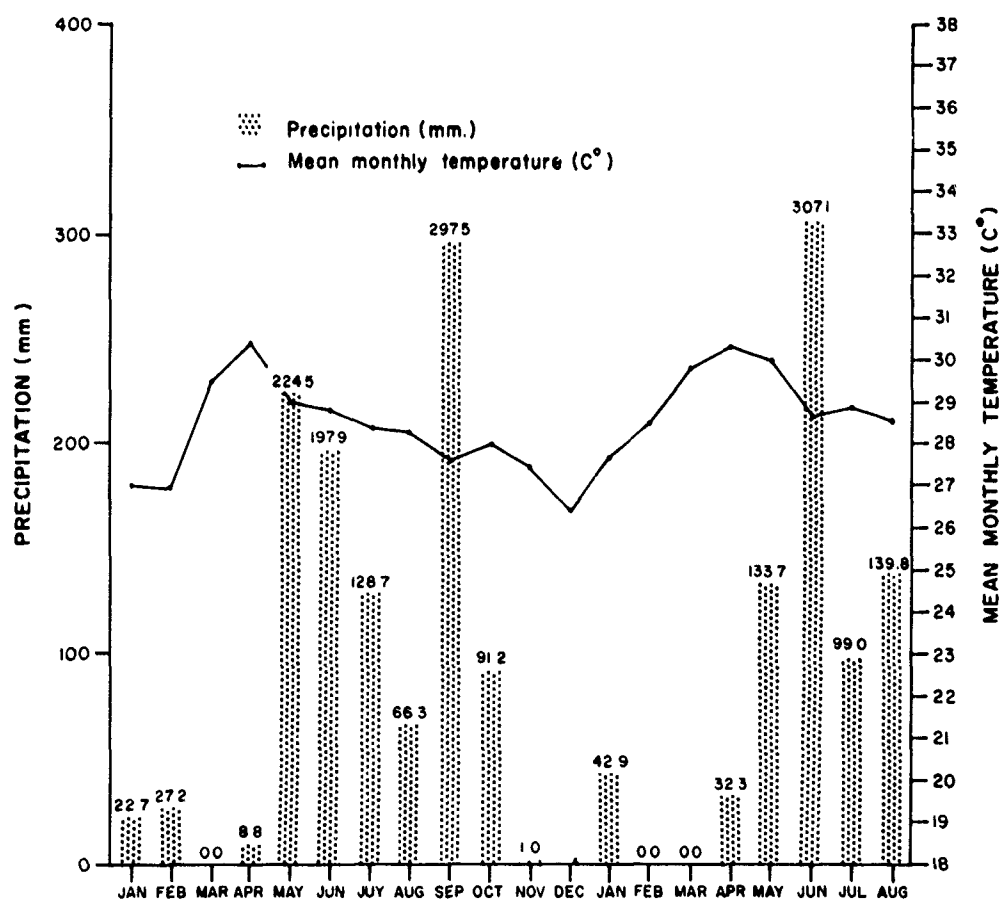


Figure 12 Precipitation¹ and mean monthly temperatures² recorded for January 1978 through August 1979.

¹ Engineering School, Meteorological Department, Ministry of Communications, Din Daeng, Bangkok, Thailand

² Climatology Division, Meteorological Department, Ministry of Communications, Bangkok, Thailand

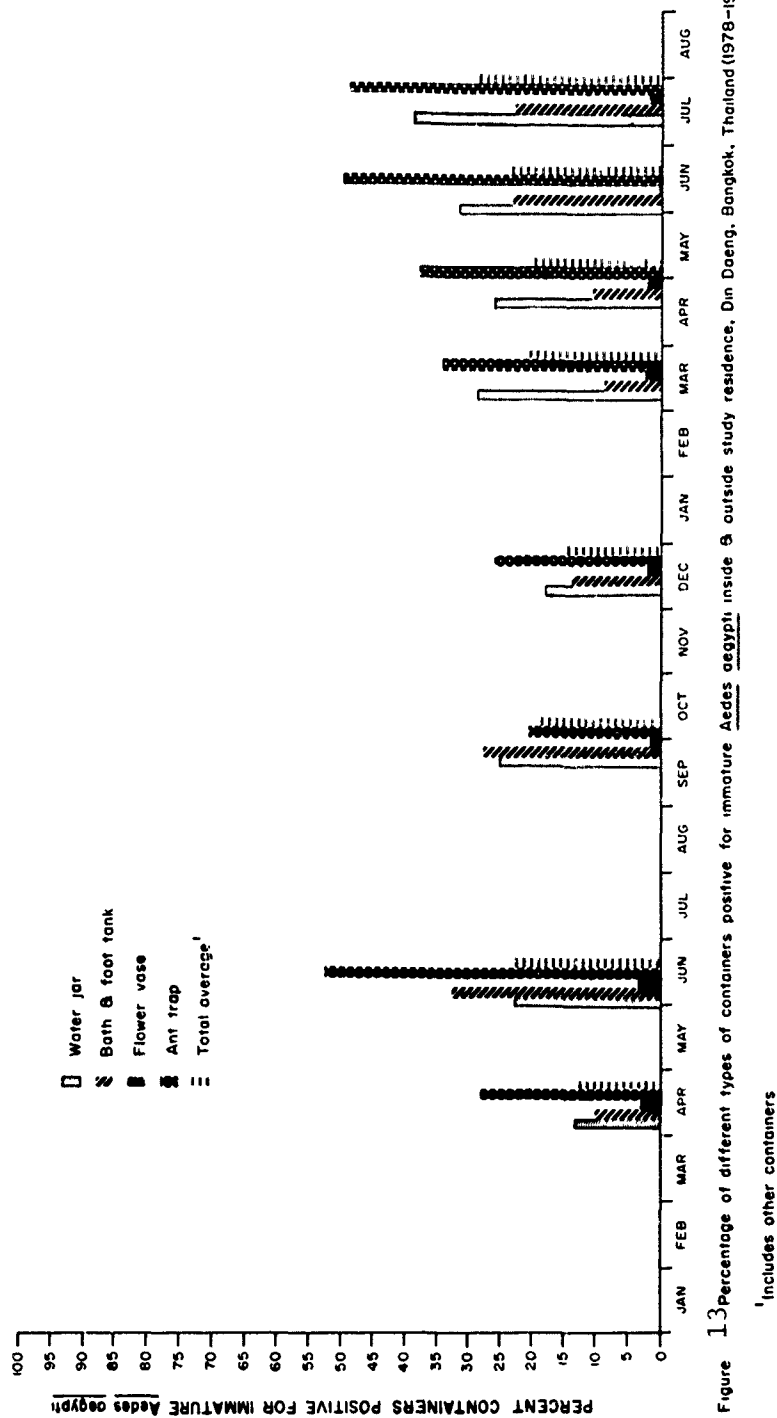


Figure 1.3 Percentage of different types of containers positive for immature *Aedes aegypti* inside & outside study residence, Din Daeng, Bangkok, Thailand (1978-1979)

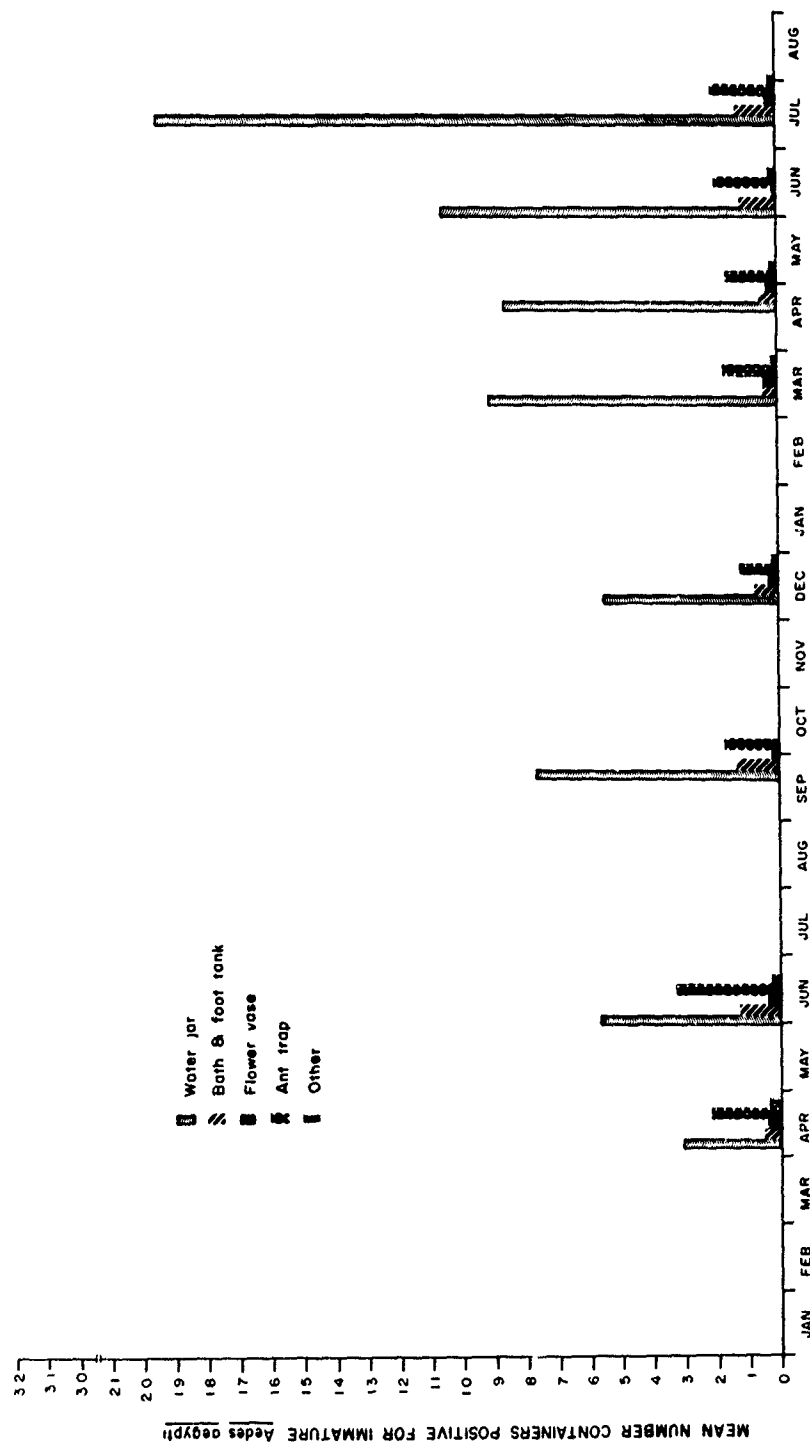


Figure 1. Mean number of containers positive for immature *Aedes aegypti* per study residence (outside & inside), Din Daeng, Bangkok, Thailand (1978-1979).

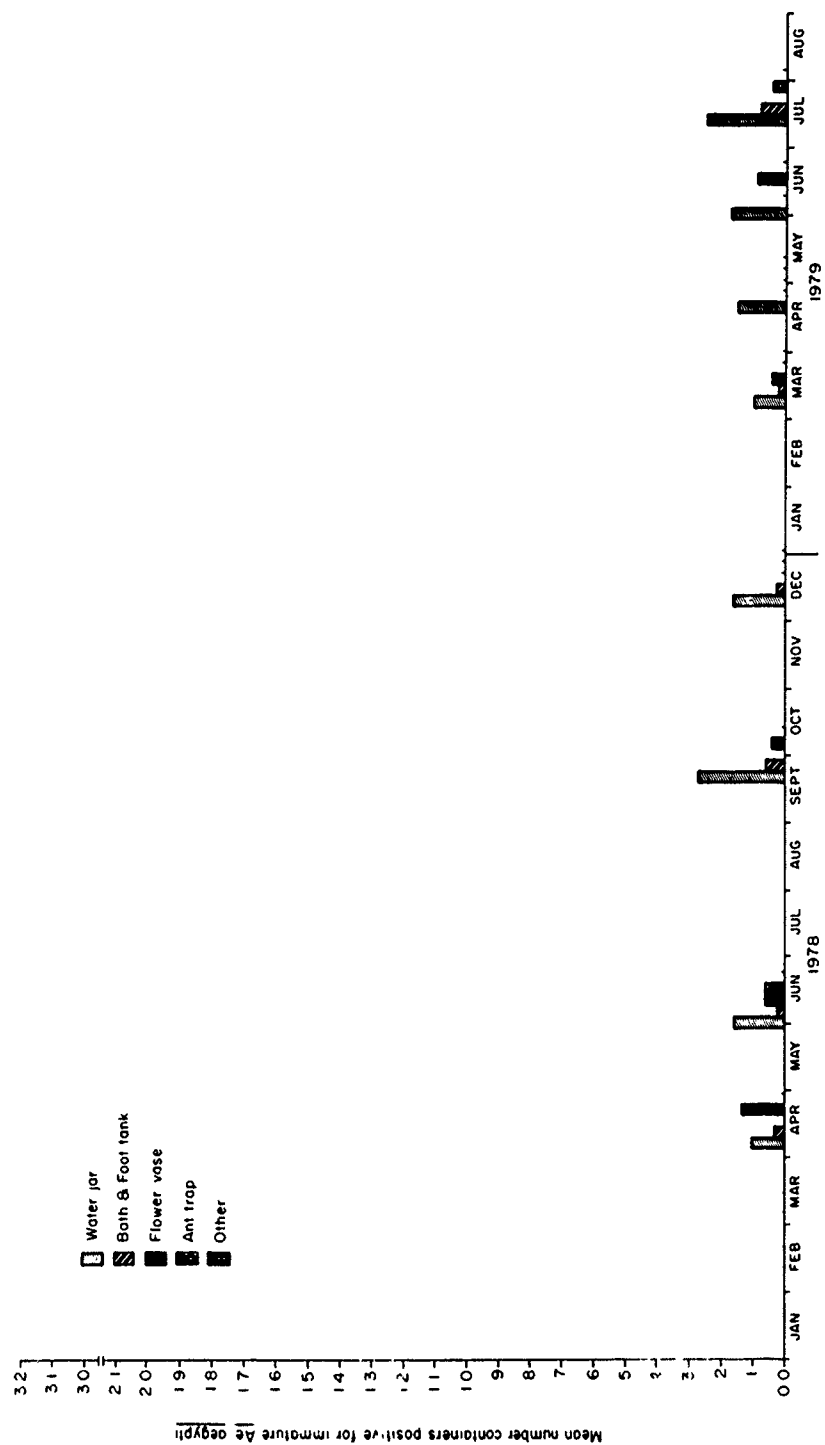


Figure 1.5 Mean number of containers positive for immature *Aedes aegypti* per high-rise flat (inside), Din Daeng, Bangkok, Thailand (1978-79)

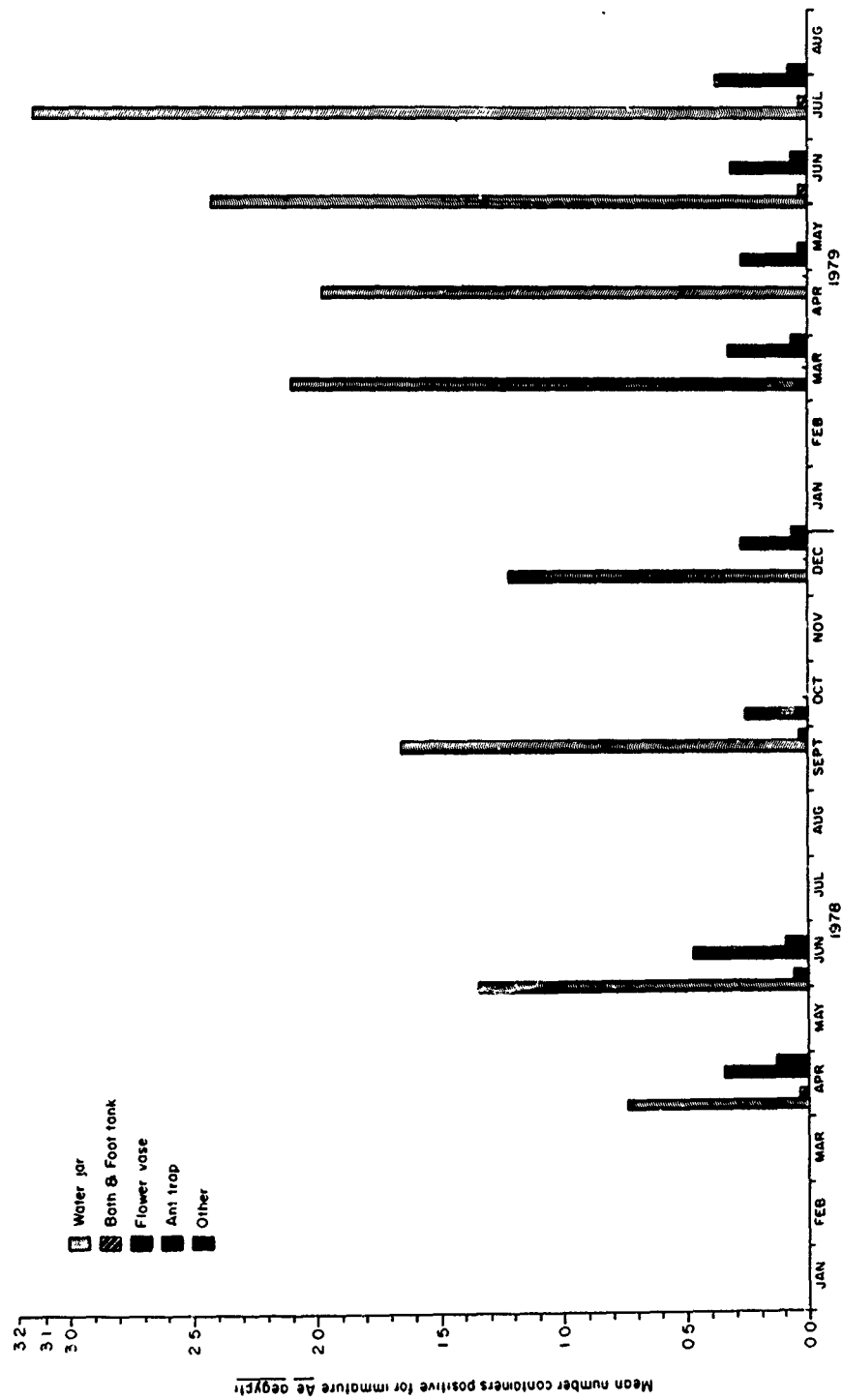


Figure 1 Mean number of containers positive for immature *Aedes aegypti* per slum residence (outside & inside), Din Daeng, Bangkok, Thailand (1978-79)

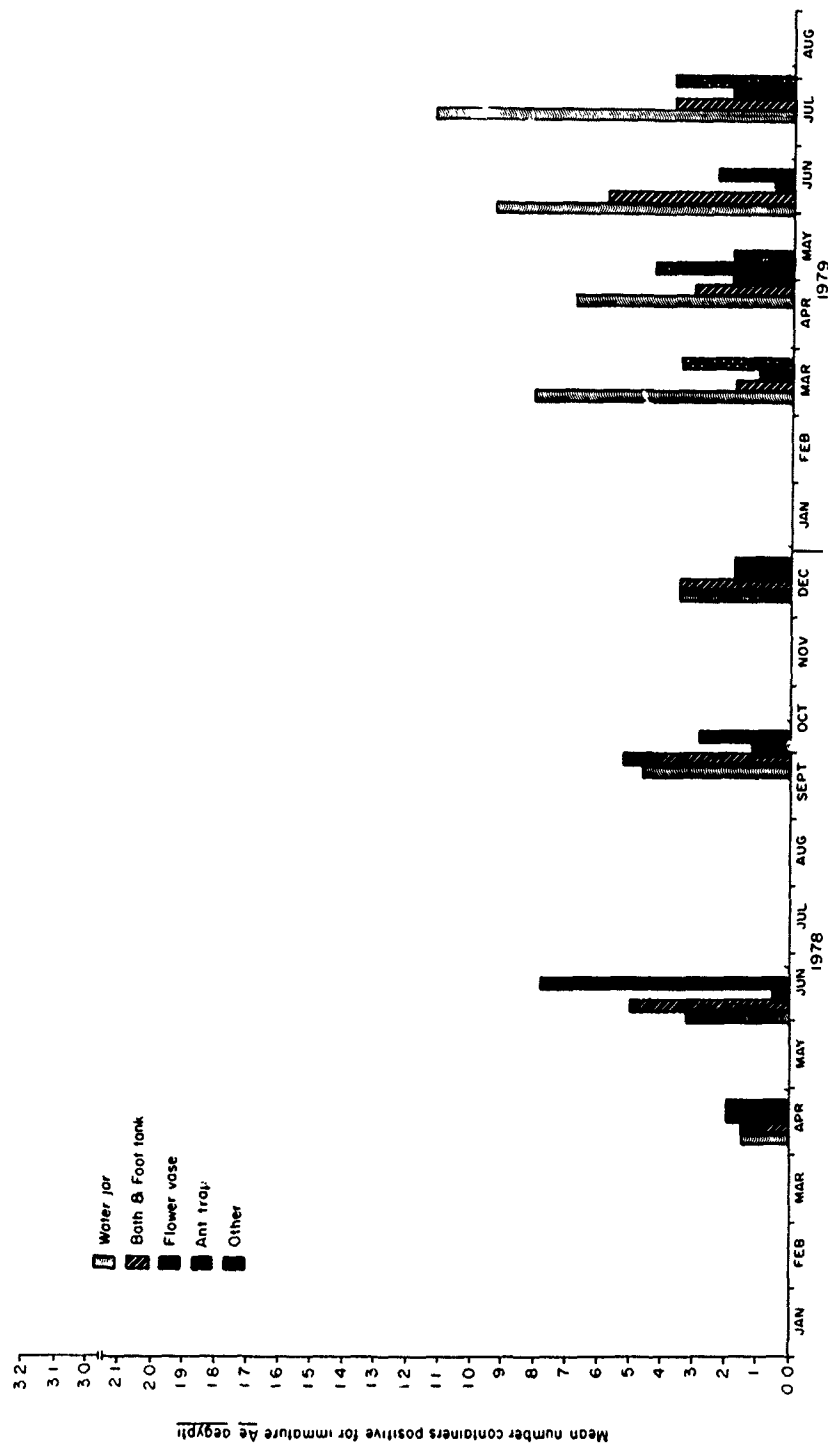


Figure 17 Mean number of containers positive for immature *Aedes aegypti* per shop residence (outside & inside), Din Daeng, Bangkok, Thailand (1978-79)

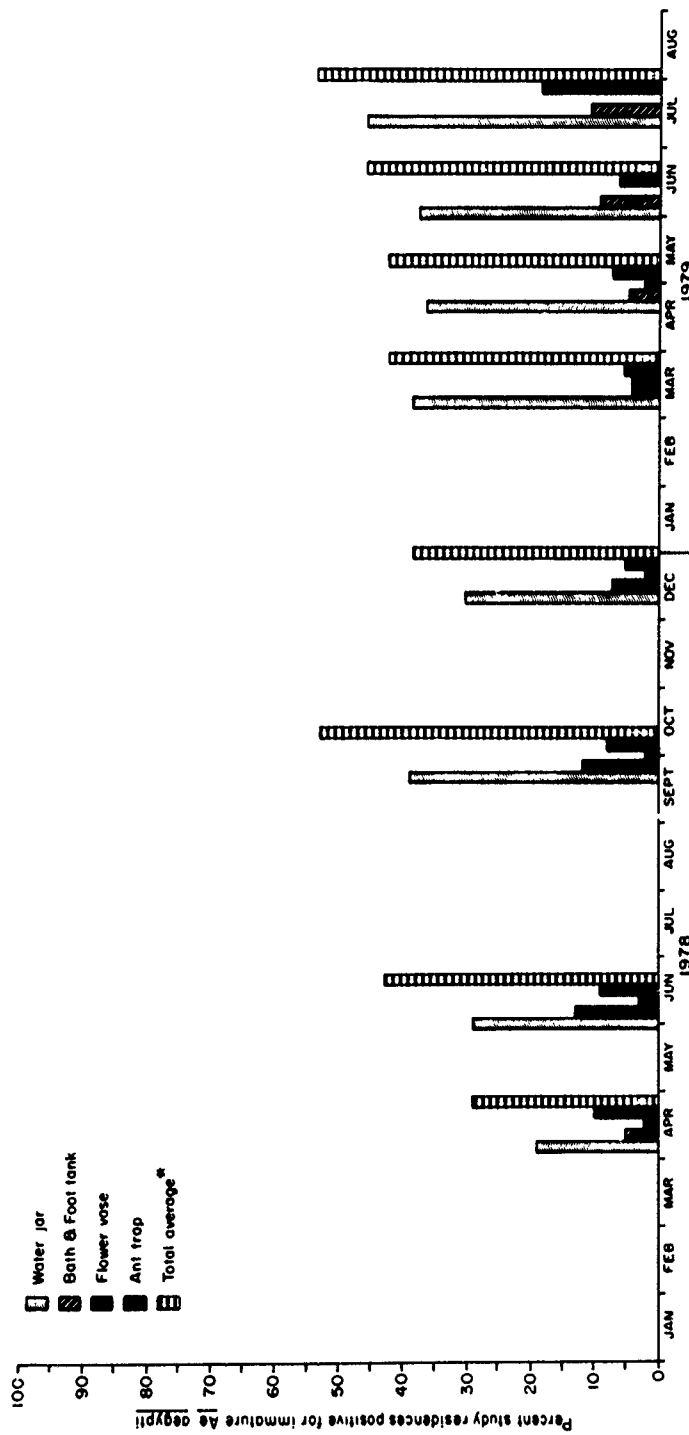


Figure 18 Percent of study residences positive for immature *Aedes aegypti* by different types of containers, Din Doeng, Bangkok, Thailand (1978-79)

* house positive for more than one type container only counted once

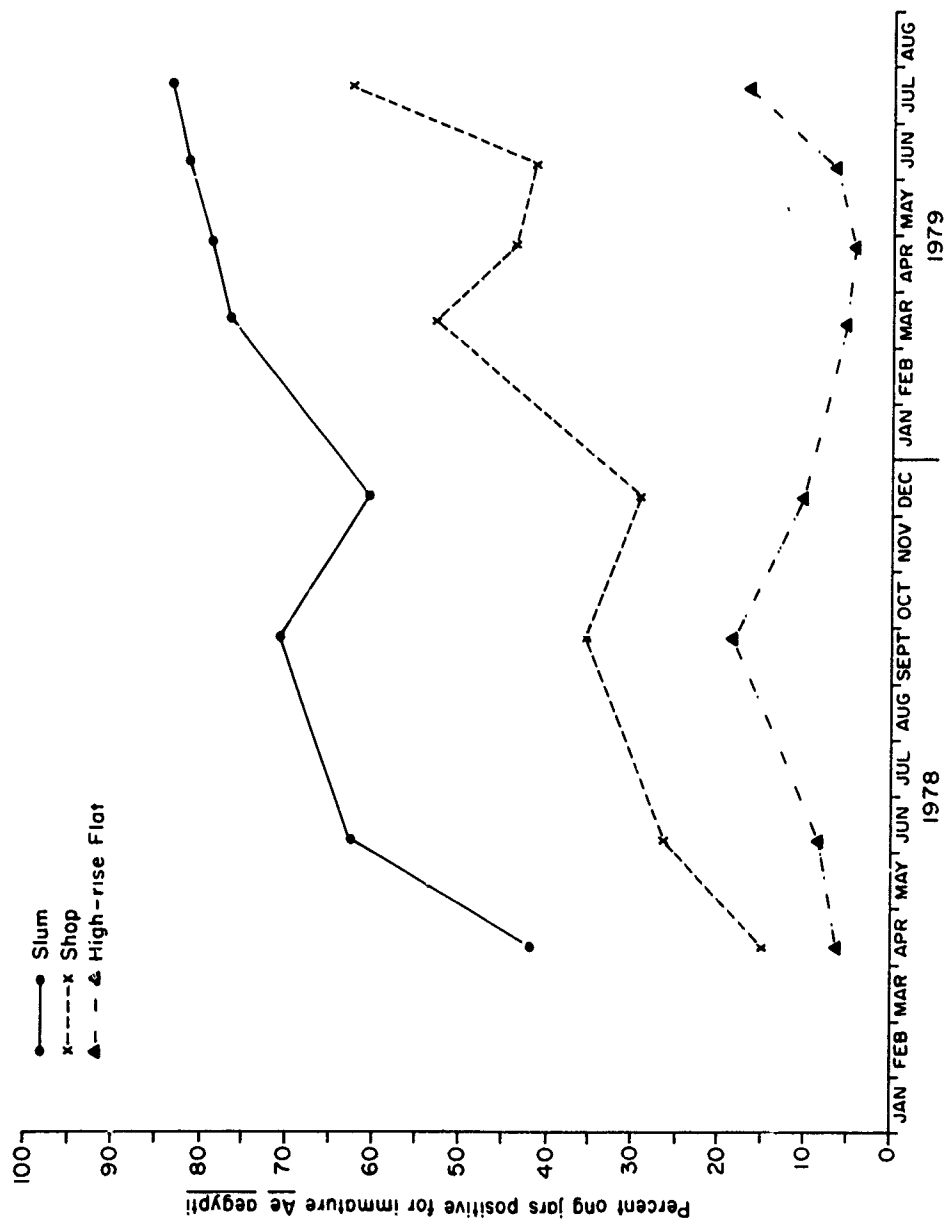


Figure 13 Percent of different type study residences positive inside & outside for immature *Aedes aegypti* for *ong* (water) jars, Din Daeng, Bangkok, Thailand (1978 - 79)

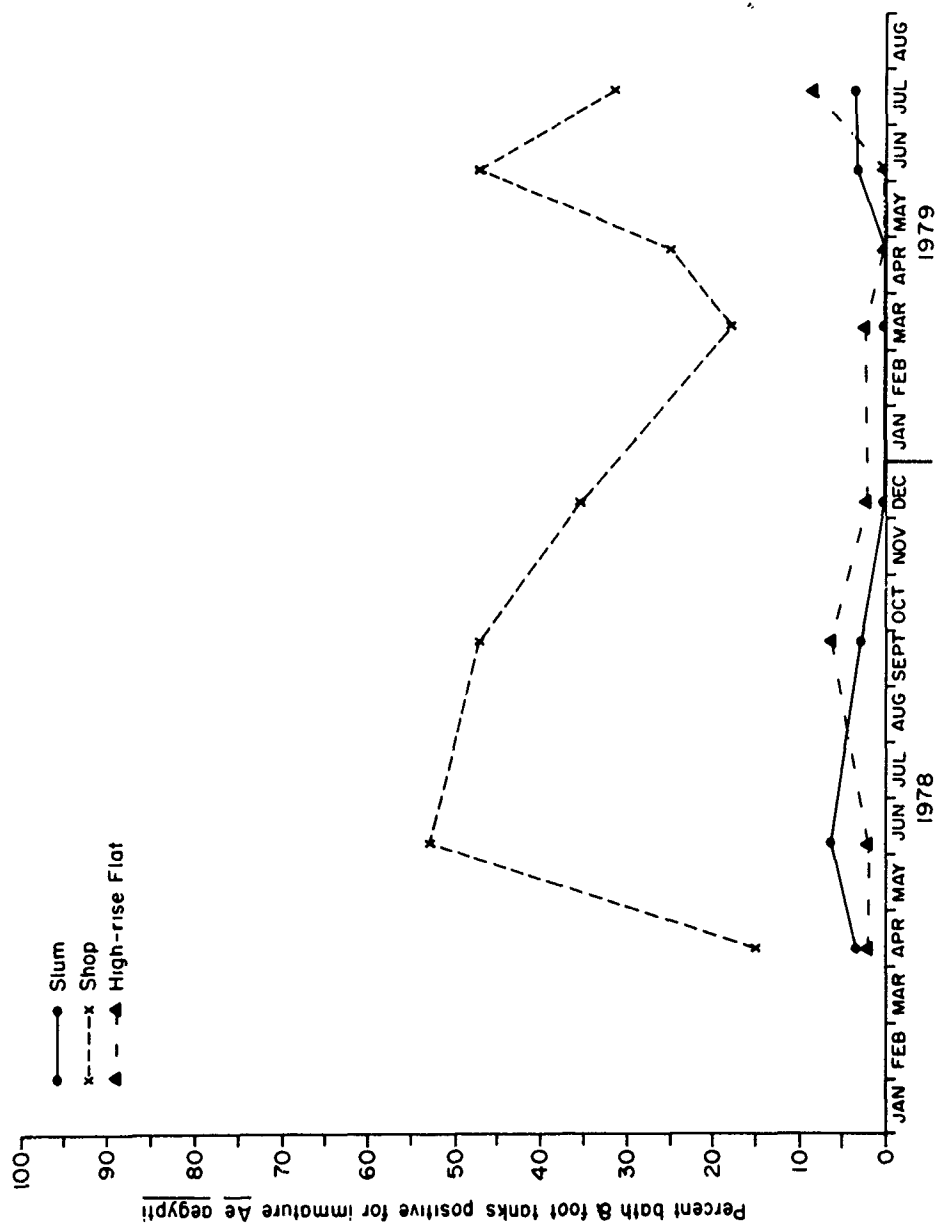


Figure 20 Percent of different type study residences positive inside & outside for immature *Aedes aegypti* for bath and foot tanks, Din Daeng, Bangkok, Thailand (1978-79)

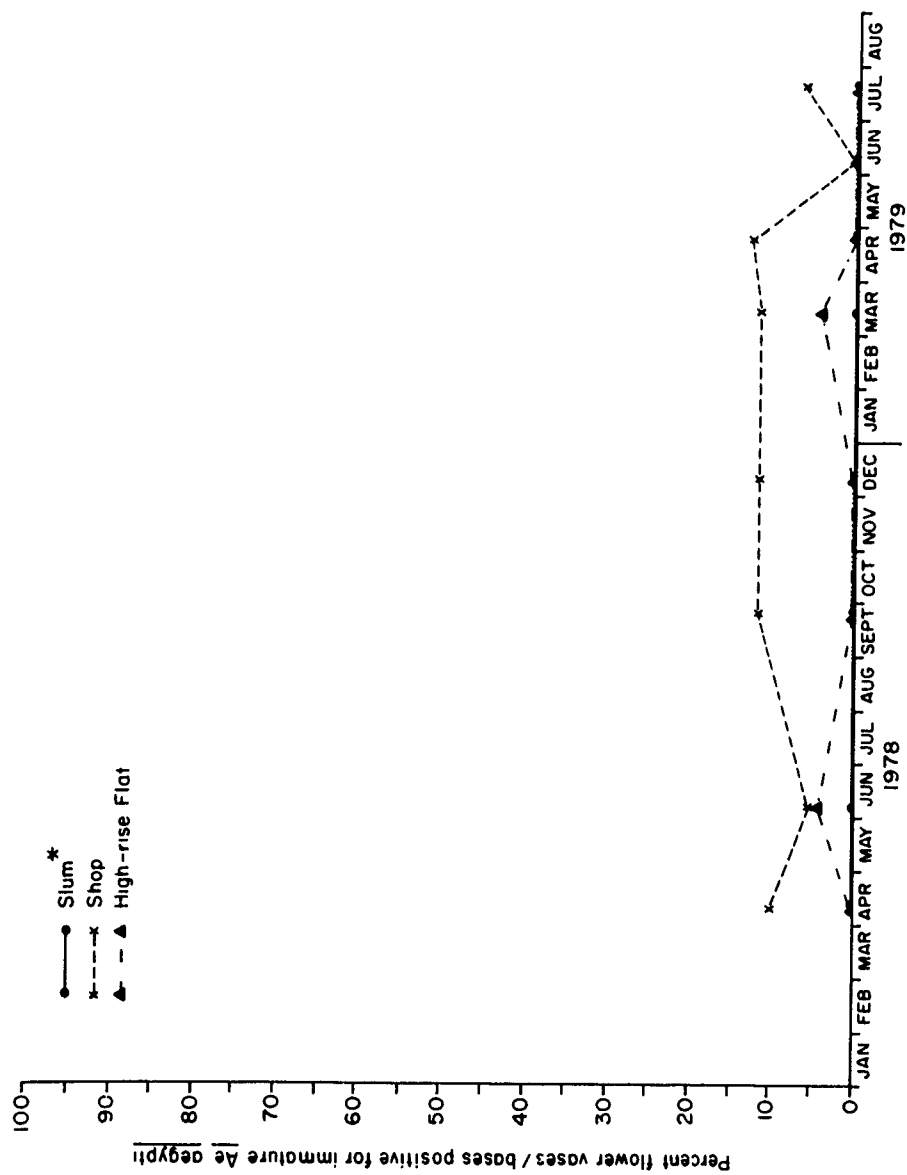


Figure 21 Percent of different type study residences positive inside & outside for immature *Aedes aegypti* for flower vases and flower pot bases, Din Daeng, Bangkok, Thailand (1978-79)

* All flower vases and pot bases observed were negative

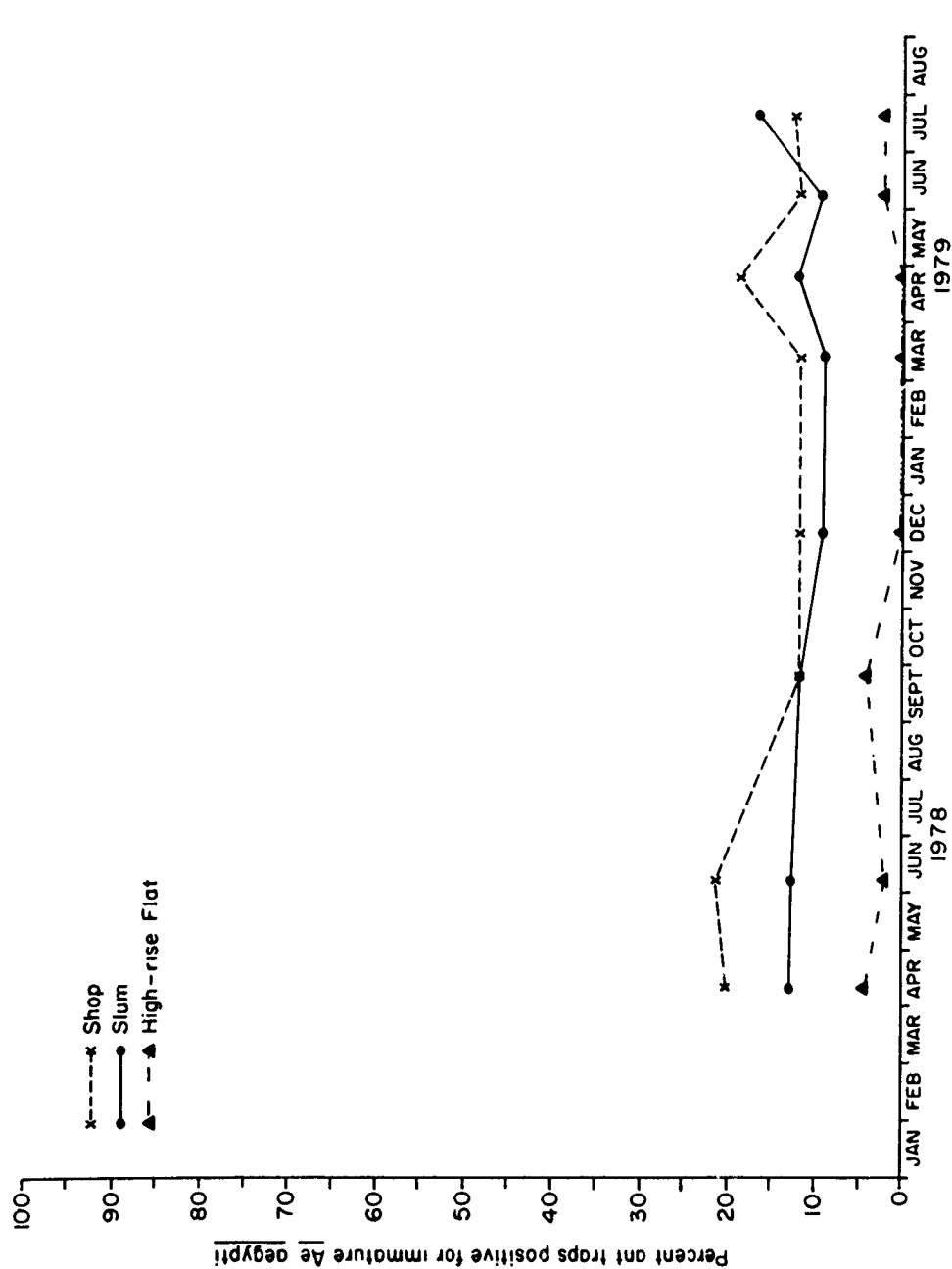


Figure 22 Percent ant traps positive for immature *Aedes aegypti* inside & outside different types of study residences, Din Daeng, Bangkok, Thailand (1978-79)

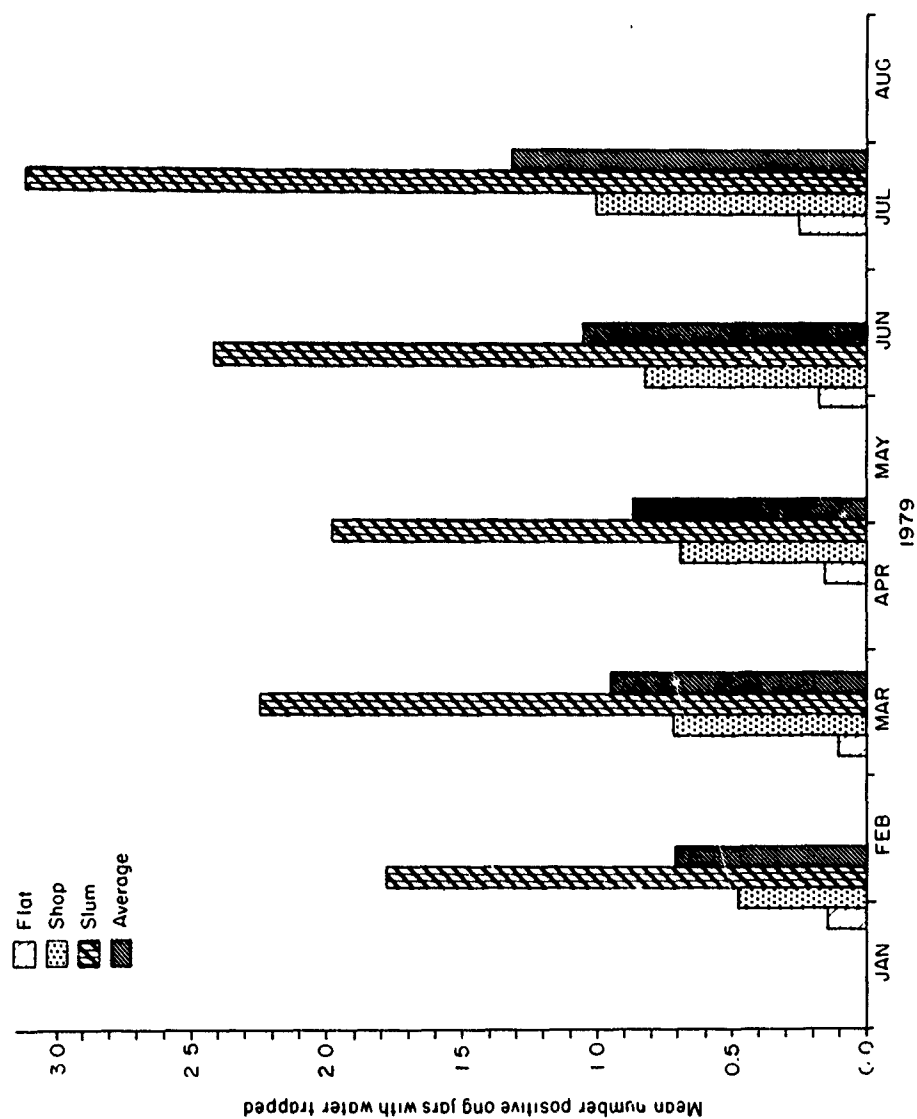


Figure 23 LARVAL TRAP Mean number of positive ong (water) jars per residences trapped with water for different study residences, Din Daeng, Bangkok, Thailand (1979)

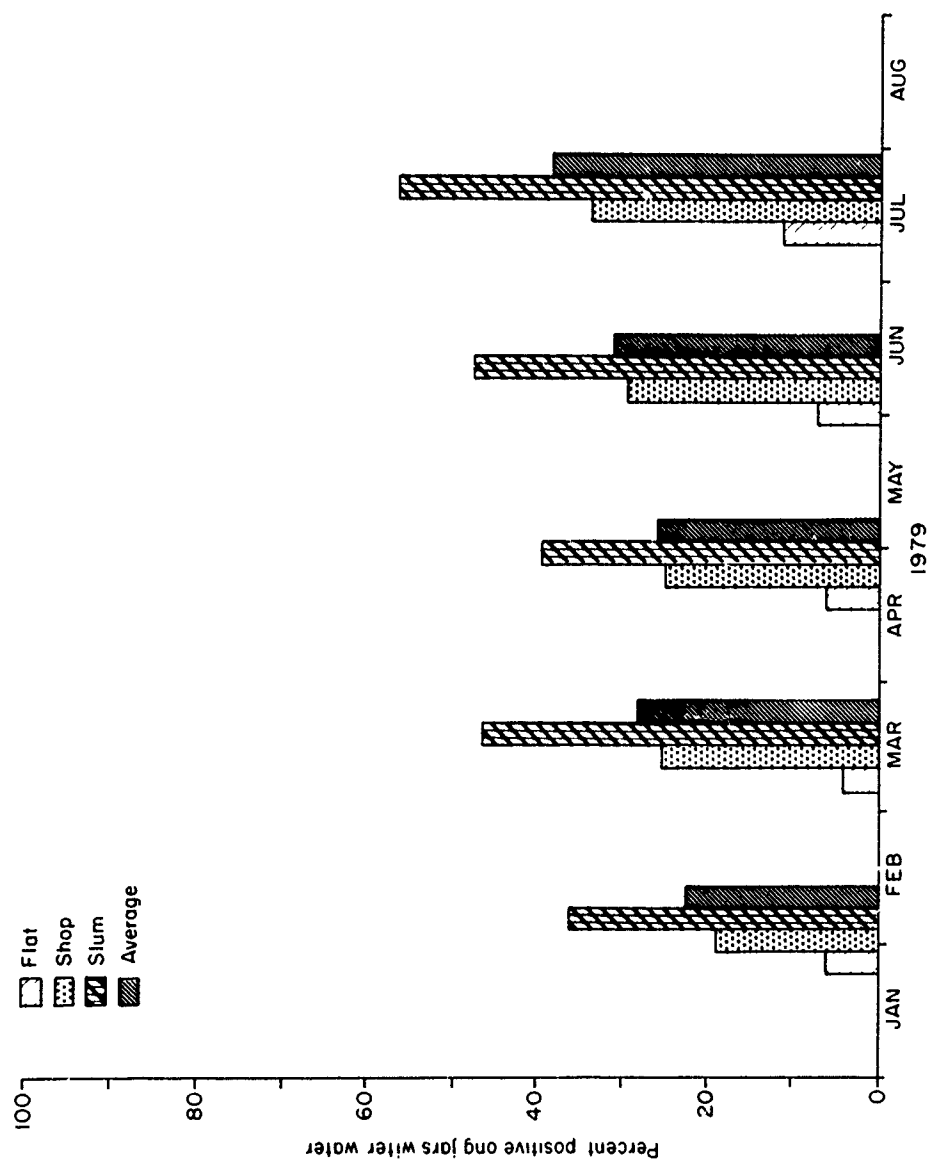


Figure 24 LARVAL TRAP Percent positive ong (water) jars trapped per total ong jars with water for study
residences, Din Daeng, Bangkok, Thailand (1979)

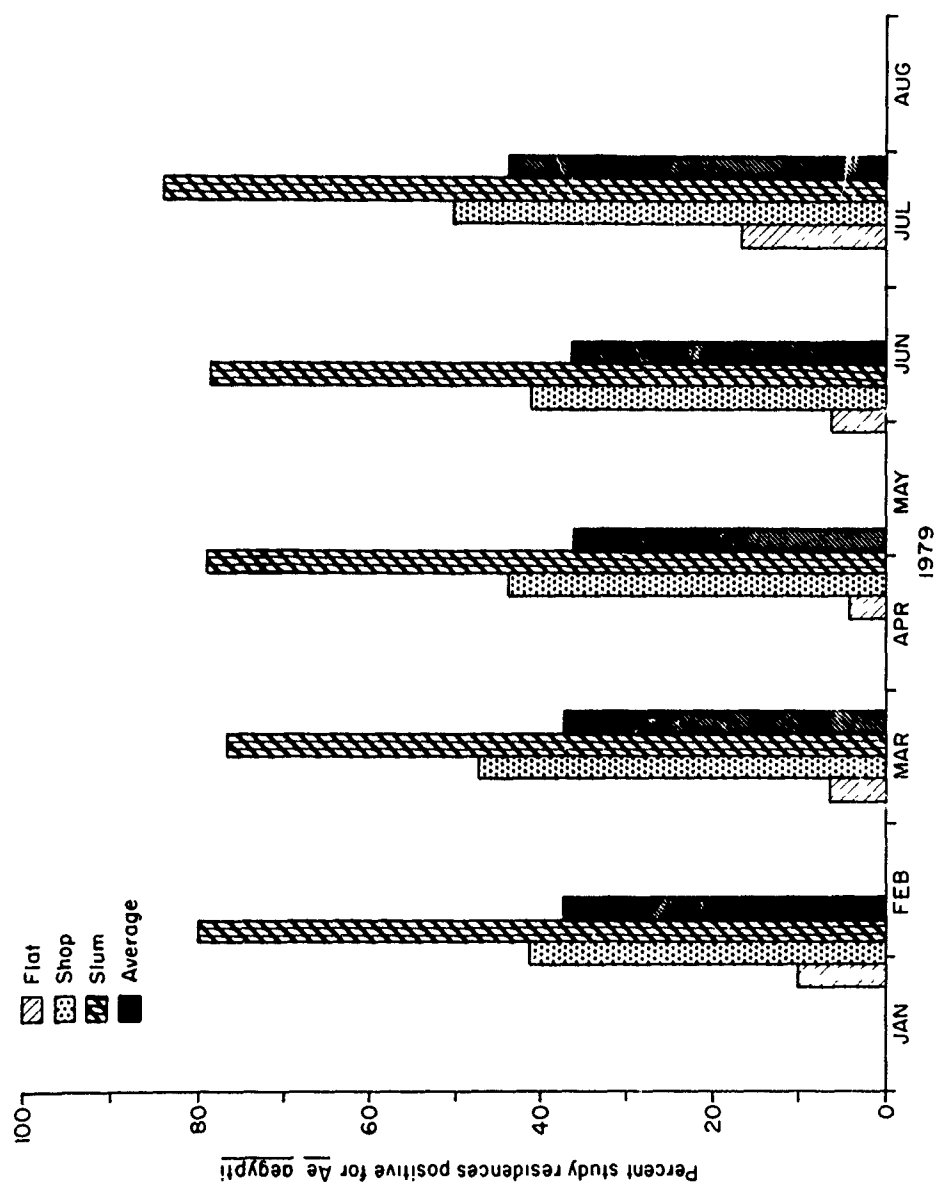


Figure 25 LARVAL TRAP Percent study residences with positive ong (water) jars that were trapped for immature mosquitoes, Din Daeng, Bangkok, Thailand (1979)

percentage of all residences positive for immature aegypti demonstrated very little change and only increased following heavy periods of precipitation (Figures 12 and 25).

These studies are continuing.

15. A Serological Survey for Togaviruses (Arboviruses)
in a Well Defined Rural Thai Population

OBJECTIVE: To study the seroepidemiology of togavirus (arbovirus) infections in a well defined rural Thai population.

BACKGROUND: As reported previously (112) sera were collected in 1975-6 from a relatively isolated rural village in central Thailand in conjunction with a malaria chemosuppressive trial. In order to assess the extent to which a rural population is subject to dengue infections, these sera were tested. This report concludes that study.

METHODS: The methods used for this study are outlined elsewhere in this report (A Longitudinal Serologic Study of a Bangkok School Population).

RESULTS: Of the total population of 1024 people in this village, sera from 638 individuals were tested. The distribution of the antibody against alphaviruses and flaviviruses are presented in Tables 38 and 39 respectively.

Because of the antigenic similarity between dengue viruses and Japanese B encephalitis virus (JEV), cross-reacting antibody to both viruses is common following a dengue infection; however, very low-titered or no dengue antibody is formed following a primary JEV infection. Of the 563 flavivirus positive individuals, only 24 (4 percent) had either an isolated JEV titer or a JEV titer at least four times greater than the highest dengue titer. Because of the small number of JEV identifiable infections and their apparent lack of clustering, the prevalence of antibodies against dengue virus and JEV were combined in Table 39.

Since many of the serum specimens had been previously examined for the presence of hepatitis B virus (HBV) antigen and antibody (113), the association between these markers of previous HBV infection and markers of togoviral infection was examined. As can be seen in Table 40 the probability that the associations noted for HBV and JEV and alphavirus are due to chance alone is very small.

TABLE 38
PREVALENCE OF ANTI-ALPHAHERPES ANTIBODY IN A RURAL THAI POPULATION

Age	MALE			FEMALE			TOTAL		
	No. tested	Alphaherpes antibody		No. tested	Alphaherpes antibody		No. tested	Alphaherpes antibody	
		No.	%		No.	%		No.	%
<1	2	0	-	3	0	-	5	0	-
2-3	17	0	-	15	0	-	32	0	-
4-5	22	1	4.5	35	2	5.7	57	3	5.3
6-7	27	2	7.4	39	3	7.7	66	5	7.6
8-9	26	3	11.5	15	0	-	41	3	7.3
10-14	45	8	17.8	60	13	21.7	105	21	20.0
15-19	32	12	37.5	34	6	17.6	66	18	27.2
20-29	41	12	29.3	67	17	25.3	108	29	26.8
30-39	29	17	58.6	46	16	34.8	75	33	44.0
40-49	24	13	54.2	22	10	45.5	46	23	50.0
≥50	15	7	46.7	22	12	54.5	37	19	51.3
Total	280	75	-	358	79	-	638	154	-

TABLE 39
PREVALENCE OF ANTI-FLAVIVIRUS ANTIBODIES IN A RURAL THAI POPULATION

Age	MALE			FEMALE			TOTAL		
	No. tested	Flavivirus antibody		No. tested	Flavivirus antibody		No. tested	Flavivirus antibody	
		No.	%		No.	%		No.	%
≤1	2	1	50.0	3	0	-	5	1	20.0
2-3	17	7	41.2	15	8	53.3	32	15	46.9
4-5	22	17	77.2	35	31	88.6	57	48	84.2
6-7	27	21	77.7	39	33	84.6	66	54	81.8
8-9	26	20	76.9	15	11	73.3	41	31	75.6
10-14	45	38	84.4	60	55	91.7	105	93	88.6
15-19	32	30	93.7	34	33	97.1	66	63	95.4
20-29	41	41	100.0	67	63	94.0	108	104	96.3
30-39	29	28	96.5	46	45	97.9	75	73	97.3
40-49	24	24	100.0	22	22	100.0	46	46	100.0
≥50	15	14	93.4	22	21	95.4	37	35	94.6
Total	280	241	-	358	322	-	638	563	-

TABLE 40

ASSOCIATION BETWEEN PREVIOUS EXPOSURE TO
HEPATITIS B VIRUS AND TOGOVIRUS AGENTS

Evidence of HBV exposure	Total villagers tested	Evidence of exposure to Togoviral agent					
		Dengue		JEV		Alphavirus	
		+	-	+	-	+	-
+	261	229	32	222	39	86	175
-	377	314	63	269	108	68	309

$$\begin{array}{llll} \chi^2 = 2.07 & \chi^2 = 15.57 & \chi^2 = 17.93 \\ y & y & y \\ \text{N.S.} & p < 0.0005 & p < 0.0005 \end{array}$$

TABLE 41

RELATIVE RISK OF CONCURRENT EVIDENCE OF JAPANESE B
ENCEPHALITIS VIRUS EXPOSURE AND HEPATITIS B VIRUS EXPOSURE

Age	Sex	Hepatitis exposure	JEV Exposure		RR*
			+	-	
<10	F	+	17	12	1.20
		-	45	47	
	M	+	14	12	1.08
		-	42	42	
>10	F	+	97	9	1.01
		-	119	12	
	M	+	94	6	1.04
		-	63	7	

RR* for: all males 1.26 all <10 1.14
all females 1.14 all >10 1.02
all villagers 1.19

RR* = Relative Risk

TABLE 42

RELATIVE RISK OF CONCURRENT EVIDENCE OF ALPHAVIRUS
EXPOSURE AND HEPATITIS B VIRUS EXPOSURE

Age	Sex	Hepatitis exposure	Alphavirus exposure		RR [*]
			+	-	
≤10	F	+	3	26	2.38
		-	4	88	
	M	+	5	21	3.23
		-	5	79	
>10	F	+	38	68	1.38
		-	34	97	
	M	+	40	60	1.12
		-	25	45	

RR^{*} for: all males 1.83 all ≤10 2.84
all females 1.78 all >10 1.29
all villagers 1.83

RR^{*} = Relative Risk

(The majority of 'JEV antibody' identified in Table 40 is probably non-specific antibody formed in response to a previous dengue infection, particularly dengue type IV). These associations appear to be age and sex dependent. Children, 10 years of age and under who had previously been infected with HBV, are at higher risk of concurrent evidence of togoviral infection than their non-HBV infected contemporaries. This increased risk appears to remain for life, but becomes diluted to some extent as a greater proportion of the entire population becomes infected with various togoviral agents. Tables 41 and 42 present the relative risk patterns of "JEV antibody" and alphaviral antibody respectively.

A manuscript of this (completed) project has been submitted for clearance.

16. A Longitudinal Serologic Study of A Bangkok School Population

OBJECTIVES:

1. To describe the prevalence of serologic evidence of previous togoviral and hepatitis B viral infections in a susceptible school population.
2. To determine the incidence of clinical and subclinical infections with the above agents during the "disease season" and during the period of the year without a large amount of clinical illness.
3. To investigate socioeconomic parameters possibly related to infection occurrence.

BACKGROUND: Togoviral Infections: Pre and post-dengue transmission season blood samples, taken June 1977 and January 1978, indicated that approximately 16 percent of the children at Phibunprachasan School experienced an infection with either a alphaviral or flaviviral togoviral agent during the 1977 "dengue season". Of 824 students who were seronegative for any flaviviral agent, 107 (13.0 percent) sustained a primary type titer rise. Of the 1055 students with at least a 1:20 titer to one or more of the flaviviruses prior to the "dengue season", 176 (16.7 percent) sustained at least one fourfold or greater titer rise between the June 1977 and January 1978 blood specimens. The corresponding values for alphaviral agents were 24 primary responses out of 1540 susceptibles (1.6 percent) and 10 infections out of the 349 students with pre-existing antibody (2.9 percent).

(

A minority of cases, clinically diagnosed as having hemorrhagic fever during the course of the school year, actually seroconverted between the seasonal blood samples (6/16).

During the "dengue season" of 1977 (approximately June to December), Bangkok sustained the highest number of hospitalized dengue hemorrhagic fever (DHF) cases recorded in Thailand (500-700/month) in seven years. In the following non-epidemic period, the number of cases fell to the usual non-epidemic level of less than 100 cases/month. During the subsequent "dengue season" beginning June 1978, the monthly total of hospitalized Bangkok patients never exceeded 200/month, and there were relatively few cases overall during that "dengue season".

METHODS: Study Design: Subsequent blood samples were collected from this population in June 1978 and January and June 1979. The June samples were taken from all children remaining at Philbunprachasan School from the original 1988 children enrolled in the study in June 1977. The January sample was only drawn on those children who were seronegative in the first three blood samples.

All blood samples obtained were tested for dengue, types I-IV, Japanese encephalitis virus and alphavirus antibodies by hemagglutination-inhibition (HI). Chikungunya virus antigen was used to test for anti-alphaviral antibodies. A sample of the positive and negative sera was chosen and tested by a microtiter neutralization test for dengue, types I-IV and Japanese encephalitis virus.

Definitions: Primary dengue infection: The acquisition of one or more type specific dengue HI antibodies in a person previously exhibiting no such antibodies (a titer of 1:20 is considered evidence of such antibody).

Secondary Dengue Infection: Evidence of an four-fold or greater titer increase in one or more type specific HI antibodies in a person exhibiting a 1:≥ 20 titer to one or more dengue antigens.

Table 43 presents the incidence of primary dengue infections between blood samples. Between January and June 1978, 14 out of 496 susceptible children (2.8 percent) sustained a primary dengue infection. Three children, including one of the above 14 children, became infected with an alphavirus (0.7 percent). Of the 482 seronegative students, 427 were retested in January 1979. Thirty students (7.0 percent) seroconverted during this "dengue season". Nineteen (4.8 percent) seronegative students acquired one or more anti-dengue antibodies between January and June 1979 during the non-epidemic period following a low level "dengue season".

TABLE 43
INCIDENCE OF DENGUE ANTIBODY ACQUISITION IN PREVIOUSLY UNINFECTED SCHOOL CHILDREN

Age	Jun 77 - Jan 78		Jan - Jun 78		Jun 78 - Jan 79		Jan - Jun 79	
	No. tested	No. (%) acquiring antibody	No. tested	No. (%) acquiring antibody	No. tested	No. (%) acquiring antibody	No. tested	No. (%) acquiring antibody
4	31	0(-)	24	1(4.2)	0	0(-)	0	0(-)
5	37	6(16.2)	22	0(-)	23	0(-)	16	0(-)
6	55	3(5.5)	31	1(3.2)	22	1(4.5)	15	0(-)
7	104	16(15.4)	70	1(1.4)	26	1(3.8)	22	0(-)
8	100	16(16.0)	65	2(3.1)	64	1(1.5)	53	5(9.4)
9	100	12(12.0)	71	1(1.4)	62	3(4.8)	53	5(9.4)
10	98	18(18.4)	73	0(-)	68	2(2.9)	62	4(6.5)
11	101	18(17.8)	60	3(5.0)	62	4(6.5)	47	2(4.3)
12	88	13(14.8)	41	1(2.4)	50	3(6.0)	26	1(3.8)
13	63	11(17.5)	15	3(20.0)	24	3(12.5)	19	0(-)
14	35	3(8.6)	17	0(-)	10	1(10.0)	8	0(-)
15	12	1(8.3)	7	1(14.3)	10	7(70.0)	4	1(25.0)
16	0	0(-)	0	0(-)	6	4(67.7)	2	1(50.0)
	824	107(13.0)	496	14(2.8)	427	30(7.0)	327	19(5.8)

TABLE 44
INCIDENCE OF DENGUE ANTIBODY TITER ELEVATION
IN PREVIOUSLY INFECTED SCHOOL CHILDREN

Age	Jun 77- Jan 78		Jan-Jun 78		Jun 78-Jan 79	
	No. tested	No. (%) demonstrating one or more elevations	No. tested	No. (%) demonstrating one or more elevations	No. tested	No. (%) demonstrating one or more elevations
4	6	1(16.7)	4	0(-)	0	0(-)
5	25	3(12.0)	24	4(16.7)	0	0(-)
6	38	8(21.1)	29	4(13.8)	2	0(-)
7	81	13(16.0)	63	4(6.3)	8	1(12.5)
8	99	23(23.2)	93	6(6.5)	9	1(11.1)
9	119	16(13.4)	110	8(7.3)	13	1(7.7)
10	144	31(21.5)	133	15(11.3)	15	3(20.0)
11	111	21(18.9)	103	21(20.4)	17	6(35.3)
12	145	19(13.1)	87	23(26.4)	7	3(42.9)
13	119	28(15.6)	83	21(25.3)	10	3(30.0)
14	83	13(15.7)	56	10(17.9)	7	2(28.6)
15	25	0(-)	18	8(44.4)	7	2(28.6)
	1055	176(16.7)	803	124(15.4)	95	22(23.2)

Table 44 presents the incidence of secondary dengue infections occurring during these intervals.

As can be seen in Table 43 there was a significant difference in the primary acquisition of dengue antibody between the "dengue season" (June 77-Jan 78) and the following non-epidemic period (Jan-Jun 78). This difference was not evident in a comparison of secondary cases (Table 44). The secondary dengue infections in the non-epidemic period were more common in older children than those infections occurring during the epidemic period, but the overall rate of secondary infection was not different during the two periods.

During the June 78-Jan 79 period, there were relatively few primary seroconversions, accurately reflecting the relative lack of hospitalized DHF cases during that period. However, secondary seroconversions continued at a rate similar to the previous two periods in the few children tested. Primary and secondary cases continued to occur predominately in the older children.

When dengue virus is not common in the population, e.g. January 1978 to January 1979, transmission tends to occur mainly in the older children. When the virus is not as rare, e.g. June 1977 to Jan 1978, transmission is not as localized. Since the mean age of admission of a child with DHF in Bangkok is less than six years, hospitalization records reflect those periods when transmission spreads to the younger age groups.

A manuscript of this (completed) project is in preparation.

17. Dengue Hemorrhagic Fever (DHF) in Bangkok: Correlation of Monthly Incidence Rates with Total Yearly Incidence Rates

OBJECTIVE: To correlate monthly incidence rates of DHF with total yearly incidence rates.

BACKGROUND: The peak incidence of reported cases of DHF in Bangkok regularly occurs during the rainy season, usually during the months on July, August, or September. The peak number of reported cases during a severe epidemic season may reach over 1000 cases per month and over 5000 cases for the entire year. Case incidence rates typically decline late in the rainy season and continue to decline during the cool season to reach a nadir usually during the subsequent February or March.

Total reported cases during a severe DHF year may be as much as ten fold greater than during a mild year, yet factors governing yearly incidence rates of DHF remain poorly understood. Although the temporal association of peak DHF incidence rates with the rainy season is self-evident, to date there has been no quantitative evidence that the amount of rain during the rainy season influences peak case rates. Other factors which may govern the severity of a given DHF season remain totally obscure.

Since the late 1950's records have been maintained of the number of cases of DHF occurring per month in the Bangkok-Thonburi metropolitan area by the Thai Ministry of Public Health.

In the early 1960's clear diagnostic criteria for DHF were established, and in April 1962 a highly efficient system of surveillance was also introduced (114).

With over 16 years of reliable monthly DHF incidence reports available to us, we sought to examine the data for evidence of an association between monthly and yearly incidence rates.

METHODS: The raw data used in this analysis came from files maintained in the Ministry of Public Health and are presented in Table 45. Ministry data were incomplete for the 16 months, September 1964 through December 1965; for these months graphic records kept by our Laboratory were the source of the number of cases reported per month.

To evaluate the data for correlations between monthly and yearly incidence rates, the monthly incidence rate for a given month was defined as the independent variable and the yearly incidence rate for that year as the dependent variable. For example, for each of sixteen March incidence rates set as independent variables (X_n), there was a corresponding yearly incidence rate set as a dependent variable (Y_n) (see Figure 26). A linear regression analysis was then performed, and the y intercept (b), slope (m) and correlation co-efficient (r) were computed. The same analysis was done for 8 months of the precedent year, 12 months of the index year, and 8 months the subsequent "DHF year."

RESULTS: Mean monthly case incidence rates for the years 1963-1978 are presented in Figure 26A, and the average ranking of the number of DHF cases per month is presented in Figure 26B. From both of these curves it can be seen that case incidence rates of DHF have a nadir in February or March; this was the basis for defining the "DHF-year" as beginning in March and ending in February.

Table 45 Cases of DHF Hospitalized per Month, April 1962-Déc 1978 - February 1979

Year	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Calendar Year Total	"DHF Year" Total*
1962	(57)	(23)	(27)	64	228	649	973 ^P	905	582	429	214	34	4185	4134**
63	21	8 ^N	14	27	108	166	306 ^P	284	288	206	115	114	1657	1857
64	103 ^N	126	210	298	417	926	1035 ^P	815	790	400	205	85	5410	5286
65	70	35	40	45	80	95	175	215	365 ^P	335	320	365	2140	2469
66	213	221	220	209	194 ^N	380	537 ^P	496	254	166	67	89	3046	2706
67	62	32	20 ^N	31	84	68	105	103	131 ^P	110	56	32	834	825
68	55	30	10 ^N	12	26	65	112	132	217 ^P	195	179	129	1661	1354
69	146	131	86 ^N	90	100	164	280	389	439 ^P	225 ^P	116	33	2199	1952
70	23	7 ^N	11	10	16	56	84	78	106	111	55	20	577	571
71	12	12	9 ^N	20 ^N	42	75	159	185	223 ^P	188	111	56	1092	1180
72	63	49	53	42 ^N	99	187 ^P	303	428 ^P	337	306	284	144	2295	2385
73	112	90 ^N	114	112	110	185 ^P	144	163	122 ^P	165	136	56	1509	1342
74	19	16 ^N	17	26	43	85	98	175	192 ^P	181	141	75	1068	1171
75	78	60 ^N	86	103	130	258	433 ^P	386	302	267	210 ^P	73	2386	2291
76	27	16	23 ^N	26	50	80	115	212	216	244	266 ^P	166	1441	1595
77	99	98	90 ^N	123	136	461	516	658	794 ^P	643	327	186	4231	4259
78	189	136	63	74	89	109	149	182 ^P	174	83	81	53	1382	1100**

P = Peak of reported cases for DHF year N = Nadia of reported cases for DHF year
 () = Monthly number of reported cases used to complete yearly total but not used in month
 by month analysis
 * Sum of number of monthly reported cases March through February of next calendar year
 (see text)
 ** Approximately value based in part on inexact or estimated monthly totals

Table 46

Month analyzed	Relationship between number of cases in month vs total number of cases during index year	r	N
Dec of preceding -DHF year	$y = 4.69 x + 1535$.321	16
Jan of preceding -DHF year	$y = 6.05 x + 1533$.292	16
Feb of preceding -DHF year	$y = 9.42 x + 1393$.464	16
Mar of Index -DHF year	$y = 12.98 x + 1157$.696	16
Apr of Index -DHF year	$y = 12.27 x + 1198$.718	17
May of Index -DHF year	$y = 11.78 x + 793$.858	17
Jun of Index -DHF year	$y = 5.07 x + 950$.931	17

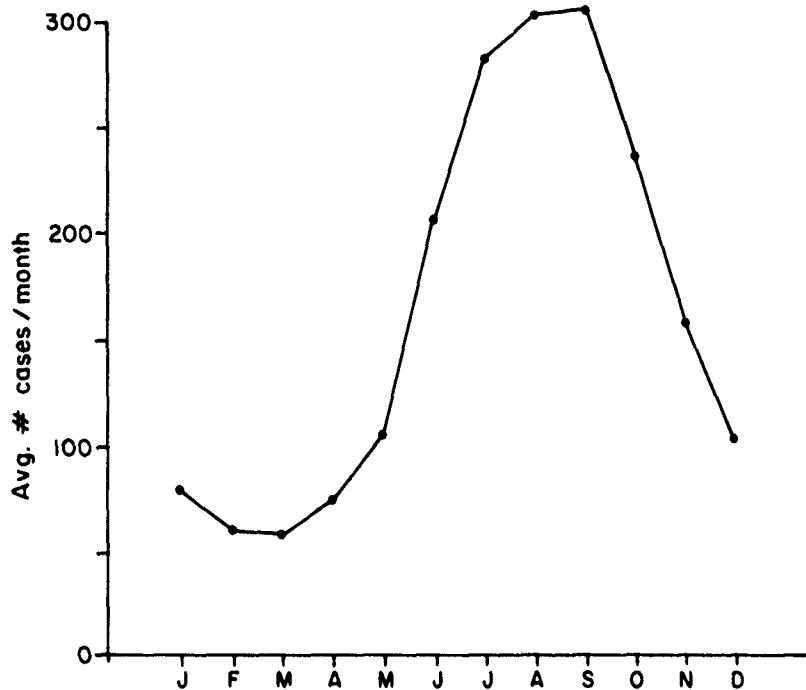


Figure 26A

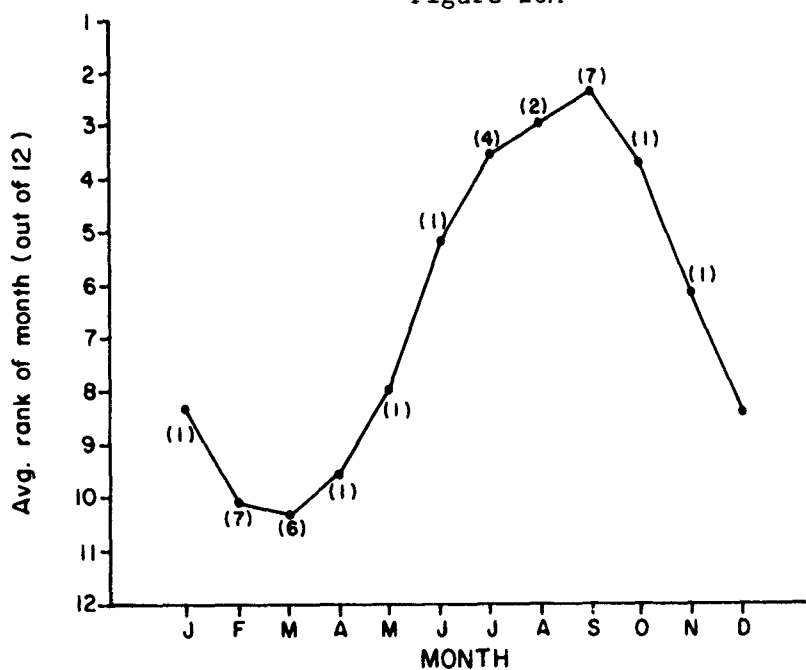


Figure 26B

Figure 26A. Graph of mean number of hospitalized cases of DHF per month, 1963-1978. Figure 26B. Graph of mean rank per calendar year of each month. Numbers in parentheses below the line represent the number of years in which the specified month had the fewest number of cases of DHF; numbers in parentheses above the line represent the number of years in which the specified month had the greatest number of cases.

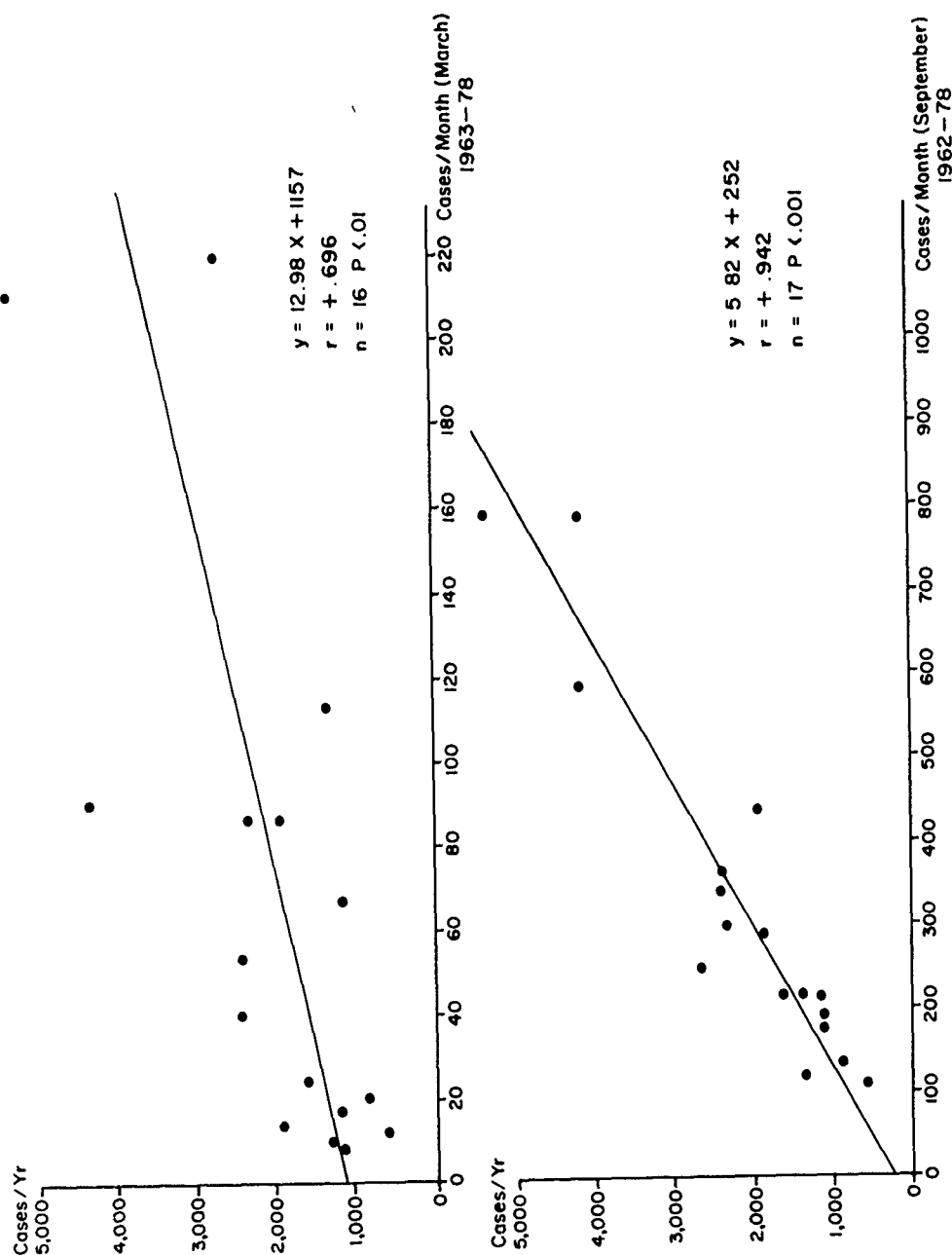


Figure 27A

Figure 27B

Figure 27A. Graph of number of cases of DHF reported each March, 1963-1978, versus the total number of cases reported that DHF year. Figure 27B. Graph of number of cases of DHF reported each September, 1962-1978, versus the total number of cases reported that DHF year.

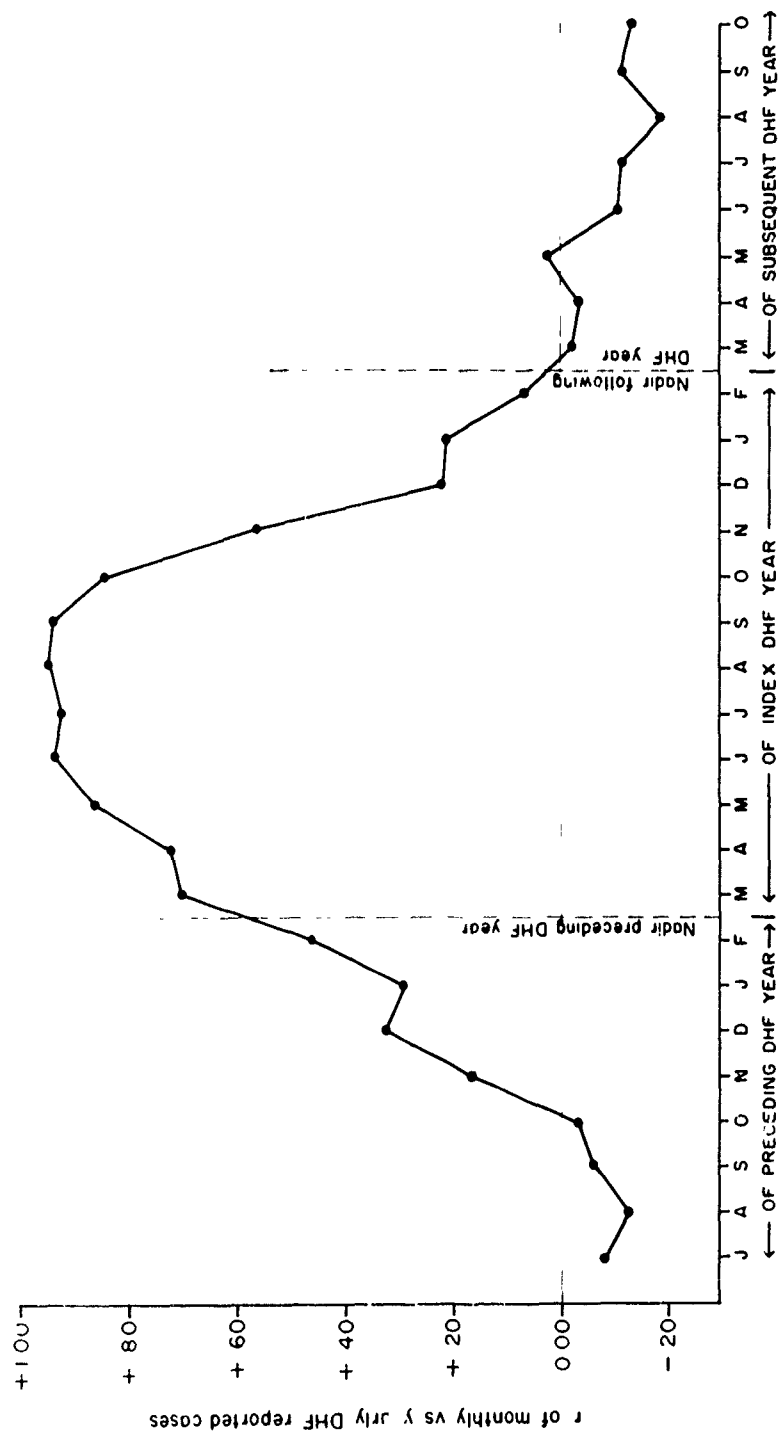


Figure 28

Graph of r (correlation coefficient) for the relationship of monthly reported DHF cases to annual total reported DHF cases.

(

As expected, the total number of cases of DHF during a given year was strongly associated with the case incidence rate during the peak months of that year. For example, the number of cases during a DHF year is plotted against the number of cases reported during the September of that year for the years 1962-1978 in Figure 27B. A correlation coefficient (r) of .942 ($p < .001$) is obtained. Correlation coefficients obtained by analysis of monthly vs "DHF-year" yearly incidence rates for 8 months of the preceding year, the 12 months of the index year and 8 months of the subsequent year, are plotted by month in Figure 28. A smooth curve is obtained with a positive slope first clearly detectable between October and February of the preceding DHF year. By March, the first month of the "DHF-year", there is already a significant ($p < .01$) correlation between the monthly and yearly incidence rates. It should be noted that the incidence rates in February and March have a much stronger correlation with the total number of cases in the year immediately following than immediately preceding these months. Restated, the case rates at the nadir of disease activity show a much stronger correlation with future, rather than past, peak incidence rates. Indeed, as far back as December, 3 months before the nadir of disease activity, case rates are more closely correlated with future, rather than past peak incidence rates, although p values do not reach the .05 level. Also, it should be noted that by the onset of the rainy season in May, the severity of the dengue season is largely determined ($r = .858$ for May vs $r = .931, .919$, and $.944$ for June, July, August respectively). The quantitative linear regression relationships between monthly and yearly DHF incidence rates for several months are presented in Table 46.

The main conclusions from this analysis are that severe DHF seasons can probably be predicted with reasonable certainty four to six months in advance of peak disease activity, and that at least some of the factors which govern the extent of disease activity during the rainy season are already well established during the preceding dry season. These factors remain as yet undetermined.

18. Fluctuations in the Relative Proportion of Dengue Virus Serotypes Isolated from Patients with Dengue Hemorrhagic Fever (DHF) in Bangkok, 1962-1978

OBJECTIVE: To summarize this Laboratory's experience in the isolation of dengue virus serotypes from patients with DHF.

Table 47 Dengue Virus Types Isolated from Metropolitan Bangkok
Hemorrhagic Fever Patients, 1962-1978

	1	2	3	4	?	Total
1962	18	23	10	1	11	63
63	6	8	17	0	1	32
64	16	49	16	2	5	88
65	0	6	0	1	0	7
66 ND						
67 ND						
68 ND						
69	1	1	3	0	0	5
70 ND						
71	3	17	1	0	0	21
72	0	14	0	0	0	14
73	5	13	4	0	0	22
74	5	6	4	0	0	15
75	1	11	7	0	0	19
76	0	8	1	5	0	14
77	0	37	11	19	0	67
78	0	31	1	5	0	37

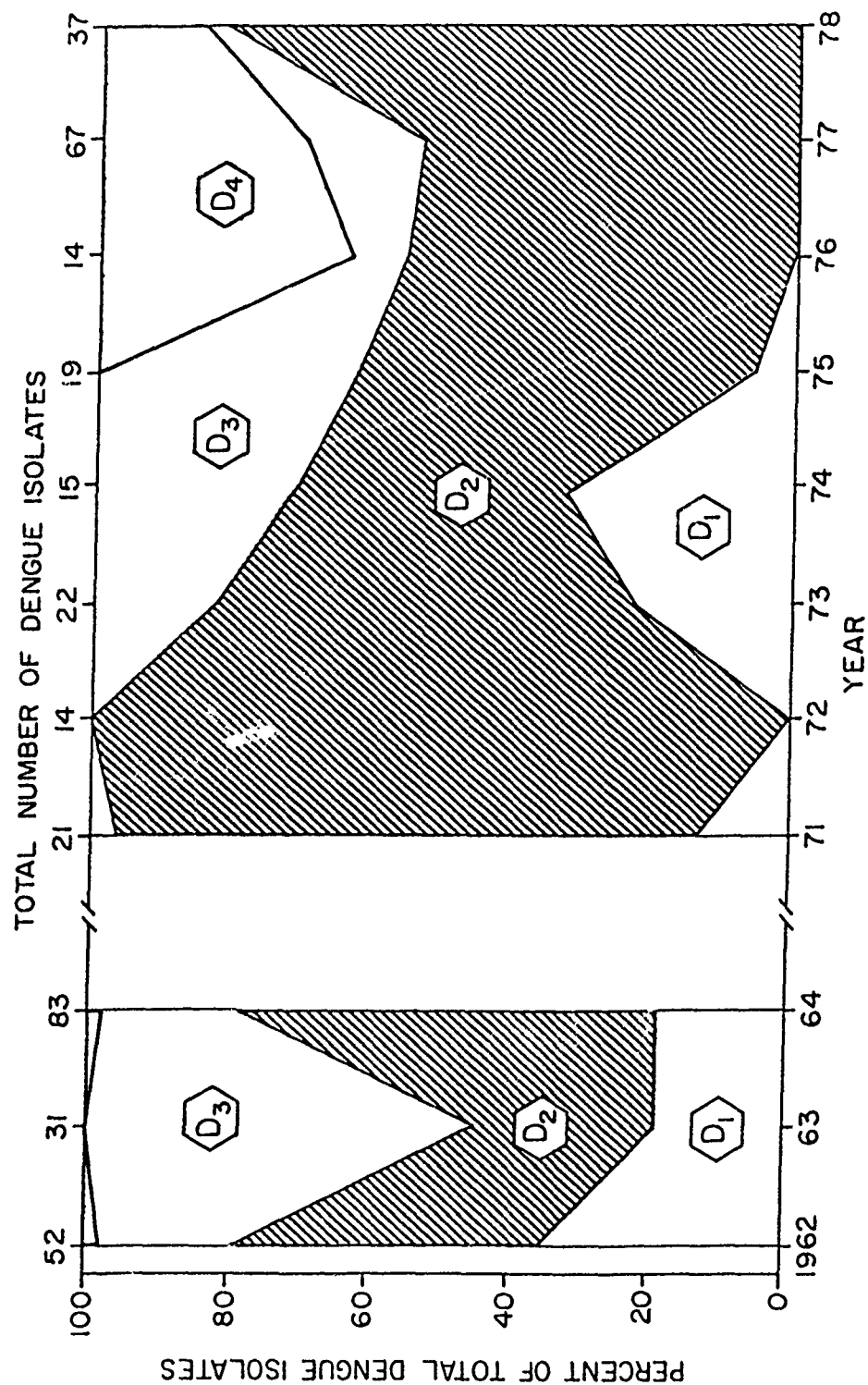


Figure 29. Isolation of Dengue Viruses, 1962-1978. Width of the Band for a given Virus Type Corresponds to the Proportion of Isolates of that Type from DHF Patients.

Table 48 Summary of Changes of Personnel, Techniques, and Reagents Used in the Department of Virology, AFRIMS (SEATO Lab) for Isolation and Typing of Dengue Viruses.

Year	Isolation Technique	Isolation Personnel	Typing Technique	Typing Personnel	Virus neutralization typing-sera: Species (f) used to raise anti-sera and reference virus			
					D1	D2	D3	D4
1962	3 passes S.M. (a)	AGM	BSC-Chik challenge (d)	Dr. A.	Mouse, proto-type	Mouse proto-type	Mouse, proto-type	Mouse, proto-type
63	"	"	"	"	"	"	"	"
64	"	"	"	Dr. A.+Nd	"	"	"	"
65	"	"	"	"	Monkey, proto-type	Monkey, proto-type	Monkey, proto-type	Monkey, proto-type
66	3 passes S.M. + Dir+Delay Plaque LLC-Mk2 (b)	Nd	Plaque on LLC-Mk2 (c)	Dr. A.	"	"	"	"
67	"	"	"	"	Monkey, 12900 (Bangkok)	Monkey, 24742 (Koh Samui)	Monkey, Ap18 (Pakistan)	Monkey, 29676 (Koh Samui)
68	"	"	"	"	"	"	"	"
69	Dir+Delay Plaque LLC-Mk2	S	"	"	"	"	"	"
70	"	"	"	"	"	"	"	"
71	"	"	"	Nd.	"	"	"	"
72	"	S&R	"	"	"	"	"	Monkey Prototype
73	"	Ny	"	"	"	"	"	"
74	"	"	"	"	"	"	"	"
75	"	"	"	"	"	"	"	"
76	"(c)	"	"	"	Monkey, CH2629-74 (Bangkok)	Monkey, CH1337-74 (Bangkok)	Monkey, VNH-73 (Vietnam)	"(g)
77	"(c)	"	"	"	"	"	"	"
78	"(c)	"	"	"	"	"	"	"

(a) Three serial passages by suckling mouse intracerebral inoculation, with mouse illness as the criterion for positivity.

(b) Direct and delayed plaque method on LLC-Mk2 cells.

(c) Isolations made from leukocyte fraction as well as plasma or serum.

(d) Log neutralization index determined with DI-D4 anti-sera on BSC-1 cells using resistance to challenge with chikungunya virus as evidence for dengue virus growth.

(e) Identification by plaque neutralization on LLC-Mk2 cells. (f) Prototype viruses are as follows: D1 (Hawaii), D2 (New Guinea C),

(g) Change in D4 reference virus made in October 1976, after the reappearance of D3-(H87), D4-(H241) dengue D4 was noted.

BACKGROUND: The Department of Virology has conducted studies on dengue virus infections since 1962. Annual Progress Reports written during this time have usually included sections detailing the total number of each of the four serotypes of dengue virus isolated during the period covered by the report. In some years, data presented in the Annual Progress Report was in incomplete form, and in no previous Annual Progress Report has the accumulated data since 1962 been compiled into a digestible summary form.

METHODS: Laboratory notebooks and records dating back to 1962 were reviewed and personnel involved were interviewed.

RESULTS: Results of the dengue virus types isolated from DHF patients in the Bangkok-Thonburi metropolitan area are presented in tabular form in Table 47 and in graphic form in Figure 29. Isolates from specimens obtained upcountry or from Koh Samui are not included. An analysis of changes in personnel, changes in techniques, and changes in reagents used for isolation and typing of dengue viruses during the period 1962-1978 is presented in Table 48.

Although many technical changes occurred during the seventeen-year period, none can be clearly associated with a subsequent change in proportion of reported serotypes.

19. Epidemiological Factors Relating to the Successful Isolation of Dengue Viruses

OBJECTIVES: To determine the serotypes of dengue viruses associated with human infections and to determine the relation of certain aspects of infection to the isolation of dengue viruses.

BACKGROUND: Epidemics of dengue hemorrhagic fever (DHF) have been documented in Bangkok, Thailand, since the early 1960's. Clinical and virological studies have shown considerable fluctuation in the types of infection and in the types of dengue viruses associated with human infections. According to data presented elsewhere in this Annual Progress Report, dengue virus type-2 has been isolated from DHF patients during every epidemic that was studied. The other serotypes have exhibited a sporadic pattern of association with human disease and the frequency of isolation has been lower than that observed for dengue virus type-2. This report describes results of virological and serological studies conducted on patients of the Children's Hospital during the 1978 dengue epidemic.

METHODS: The subjects of this study were patients treated at the out-patient clinic and/or admitted to the Children's Hospital, Bangkok, during 1978. Out-patients had febrile illnesses and admitted patients had signs and symptoms consistent with those of dengue and/or dengue hemorrhagic fever (DHF). The day of onset of fever was recorded as the first day of disease. Heparinized blood (7 ml) was obtained from patients during acute phase of illness and a second blood sample (3 ml) was obtained, when possible, 2 to 3 weeks later. Clinical histories of patients were recorded on standardized forms.

One ml of blood per patient was submitted for hemogram analysis and the remainder was centrifuged at 2000 rpm for 15 minutes. Each plasma fraction was transferred to each of 2 vials, and then stored at -20°C and -70°C for serological and for virological testing, respectively. The cellular fraction was separated into platelet and leukocyte subpopulations for virus assay as described elsewhere (23).

Plasma and cellular fractions were assayed for virus by the mosquito inoculation technique employing Toxorhynchites splendens, the direct and delayed plaque assay and an infectious center assay using LLC-Mk2 cells (23). Viruses were identified by plaque reduction neutralization tests employing dengue virus types 1, 2, 3, and 4 monospecific antisera prepared in rhesus monkeys.

Acute and convalescent plasma of patients was acetone extracted and tested for (HI) antibody by the micro-hemagglutination inhibition test. Acetone extracted mouse brain antigens of dengue virus types 1, 2, 3 and 4 were used in the test, 8 hemagglutinating units per antigen. Confirmation of dengue virus infection of patients was based on a clinical diagnosis of dengue and/or DHF accompanied by a virus specific immune response and/or isolation of virus as described in results section (Table 49).

Except for modifications presented in results section, the diagnostic criteria employed for classification of the type of infection were those of the World Health Organization (WHO) (115). Illnesses with an antibody titer of <20 before the 4th day of disease and a four-fold or greater increase in titer, but not greater than 1:1280 one to 4 weeks later, were classified as primary infections. A secondary infection was recorded for patients with antibody titers <20 in plasma obtained before the 5th day of disease with a titer >2560 in convalescent plasma, or titers >20 in plasma obtained before the 5th day after onset of disease, with a four fold or greater increase in titer for

convalescent plasma. An HI antibody titer of >1280 in acute and convalescent plasma without a four fold difference was considered to be a presumptive secondary infection.

RESULTS: A total of 178 patients who were examined at the hospital and/or the out-patient department during 1978 were included in virus isolation studies. Of the 178 patients, 138 were clinically diagnosed dengue fever and/or dengue hemorrhagic fever cases. The remaining 40 were patients with febrile illness, the majority of whom were selected from the out-patient department of obtain preliminary data and/or virus isolations for other investigations. Of the 138 patients with a clinical diagnosis of DHF, presumptive or definite evidence of dengue virus infection was found by virus isolation and/or by serology in 118 cases. Of the 20 cases with unproven dengue etiology, paired plasma for diagnostic tests were available for 11. A correct diagnosis was thus made for 91.5% (118/129) of the patients. Dengue virus infections was diagnosed in 38% (10/32) of the patients with undifferentiated pyrexia with paired plasma specimens. A summary of the distribution of the 178 patients by etiology and clinical presentation of illness is presented in Table 49.

One hundred and twenty-four of the 128 confirmed or presumptive dengue virus infected patients were classified as primary or secondary infections. Table 50 presents the distribution of the types of infections by sex and age. On the basis of the criteria employed, only two patients (1.6%) had primary infections. An additional five patients (2 males and 3 females) had possible primary infections. Studies are in progress to confirm the type of infection. One hundred and twenty-two patients (95.3%) had secondary infections and 4 (3.1%) infections could not be classified. The unclassifiable infections were based on the isolation of dengue viruses from unpaired plasma specimens with antibody titer for 3 of 4 equal to or less than 1:20.

No apparent difference was noted between male and female for the different types of infections. The age of dengue virus infected patients ranged from less than one year to 16 years of age, with infections being most common in the 4 to 7 year age group (Table 51)

Table 52 summarizes the types of dengue viruses isolated from plasma and cellular components of the blood of dengue patients by type of infection. Seven additional isolates were detected, but were lost while attempting to increase infectivity titers for identification tests. Dengue virus types 2, 3 and 4 were isolated with 86.5% of the isolates being dengue virus type 2.

Table 49 Distribution of 178 Dengue Patients by Etiology of Illness.

Etiology	Number of of Cases	Diagnostic criteria
Dengue viruses	64	Four-fold or greater difference in dengue virus HI antibody titer between acute and convalescent plasma with or without virus isolations (24 virus isolations)
Dengue viruses	13	Isolations of dengue viruses from patients for whom serological results did not meet confirmed dengue virus infection criteria.*
Presumptive dengue viruses	26	Dengue virus HI antibody titer $\geq 1:1280$ in paired plasma for one or more serotypes without 4 fold increase in titer
Presumptive dengue viruses	25	Dengue virus HI antibody titer $\geq 1:1280$ in unpaired plasma for one or more serotypes.
Not dengue	28	Paired plasma specimens that did not meet any of the above criteria.
Not dengue	22	Unpaired plasma specimens that did not meet any of the above criteria.
Total	178	

* Four virus isolations from unpaired plasma, HI antibody titer $\leq 1:80$, 5 virus isolations from unpaired plasma, HI antibody titer $\geq 1:1280$ and 4 virus isolations from paired plasma, HI antibody titer $\geq 1:1280$ without 4 fold increase in titer.

Table 50 Distribution of Primary and Secondary Dengue Virus Infections by Sex.

Type of infection	Males		Females		Total	% of total Cases
	Number	%	Number	%		
Primary	01	50	01	50	02	1.6
Secondary*	59	48	63	52	122	95.3
Unclassified	01	25	03	75	04	3.1

* Includes 5 possible primary infections (2 males and 3 females), acute antibody titer $\geq 1:20$ on days 5 and 6 of illness with a four fold increase in titer, but $\leq 1:640$ (WHO criteria) includes 30 cases that did not meet WHO criteria, 5 (1 male and 4 females) patients whose unpaired plasma specimens contained antibody titers $\geq 1:1280$ plus virus isolations, and 25 (14 males and 11 females) patients whose unpaired plasma specimens contained antibody titers of $\geq 1:1280$ to one or more dengue virus sero-type.

Table 51 Distribution of Primary and Secondary Dengue Virus Infections by Age.

Age	Primary	Secondary	Unclassified Confirmed	Total	% of total Cases
0-3	01*	26	03	30	23
4-7	00	51	00	51	40
8-11	01	27	01	29	23
12-15	00	17	00	17	13
16	00	01	00	01	01
Total	02	122	04	128	100

* Two years old.

Table 52 Dengue Virus Isolations from Dengue Patients by Sero-
type and the Type of Infection.

Type of Infection	Number of Patients	Number of Isolates	Isolation Rate (%)
Primary	02	1*	50
Secondary	122	32**	26
Unclassified	04	04***	100
Total	128	37	29%

* Dengue virus type-2

** Dengue virus type-2 (27), dengue-4 (4), dengue-3 (1)

*** Dengue virus type-2

Table 53 Distribution of Dengue Virus Isolations by Age and Sex of Dengue Patients.

Sex	Age (Years)							Total
	0-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16
Males (%)	29 (2/7)	27 (3/11)	10 (1/10)	55 (6/11)	22 (2/9)	38 (3/8)	40 (2/5)	00 (0/0)
Females (%)	00 (0/10)	40 (6/15)	19 (3/16)	17 (1/6)	43 (3/7)	44 (4/9)	33 (1/3)	00 (0/1)
Total (%)	12 (2/17)	35 (9/26)	15 (4/26)	41 (7/17)	31 (5/16)	41 (7/17)	38 (3/8)	00 (0/1)
								30 (37/128)

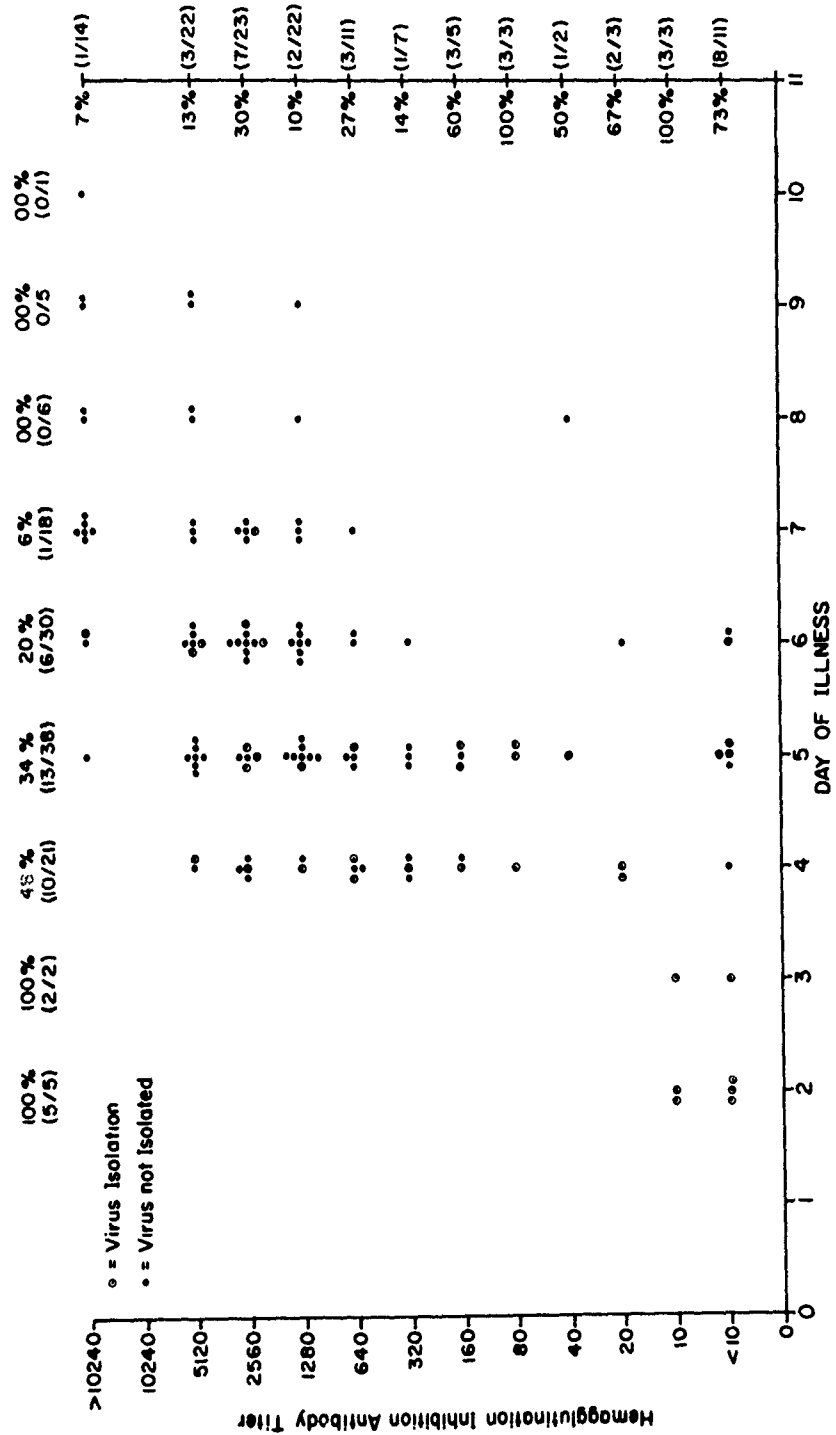


Figure 30 Distribution of dengue virus isolation in relation to hemagglutination inhibition antibody titer and of the day of illness of dengue patients

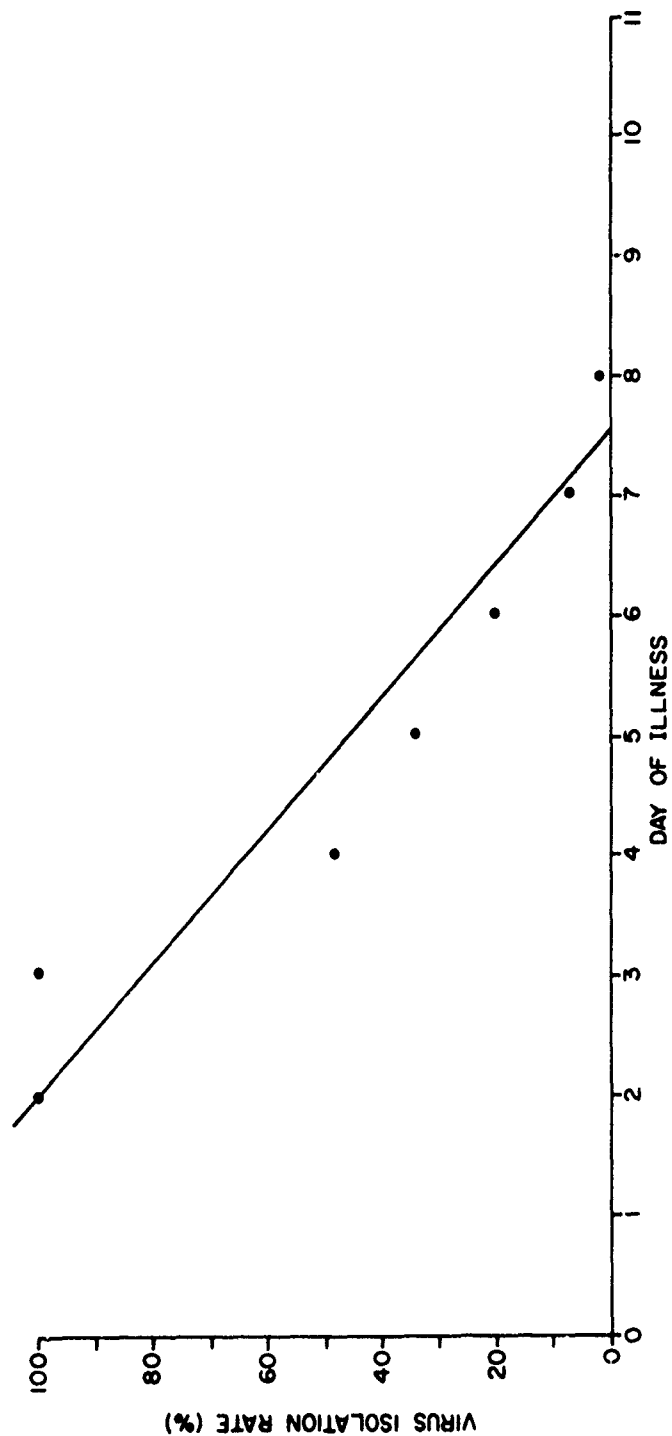


Figure 31 Correlation of the isolation rate of dengue viruses with the day of illness of dengue patients ($r = 0.958$, $p = 0.001$)

Distribution of Dengue Virus Isolations in Relation to Hemagglutination Inhibition Antibody Titer and of the Day of Illness of Dengue Patients.

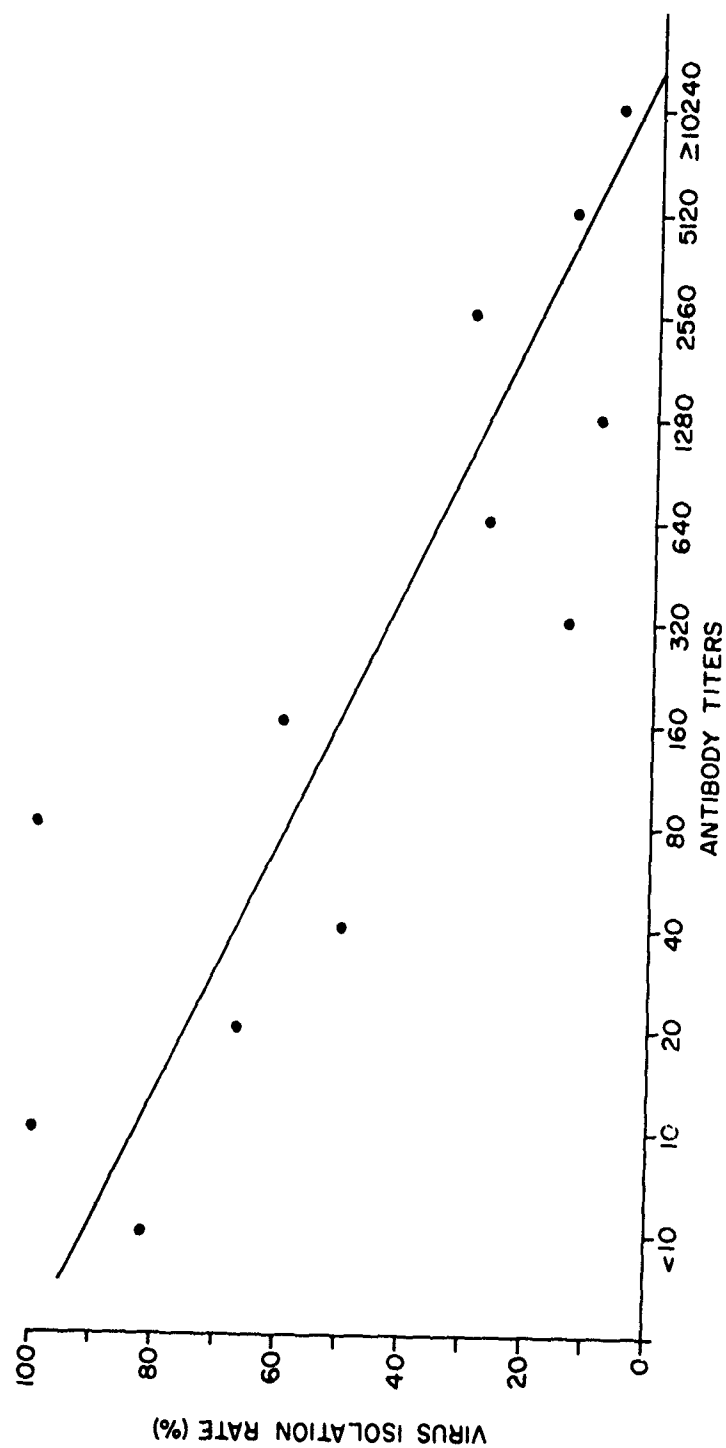


Figure 32 Correlation of the isolation rate of dengue virus with hemagglutination antibody titer of dengue patients
($r = 0.843$, $p = 0.001$)

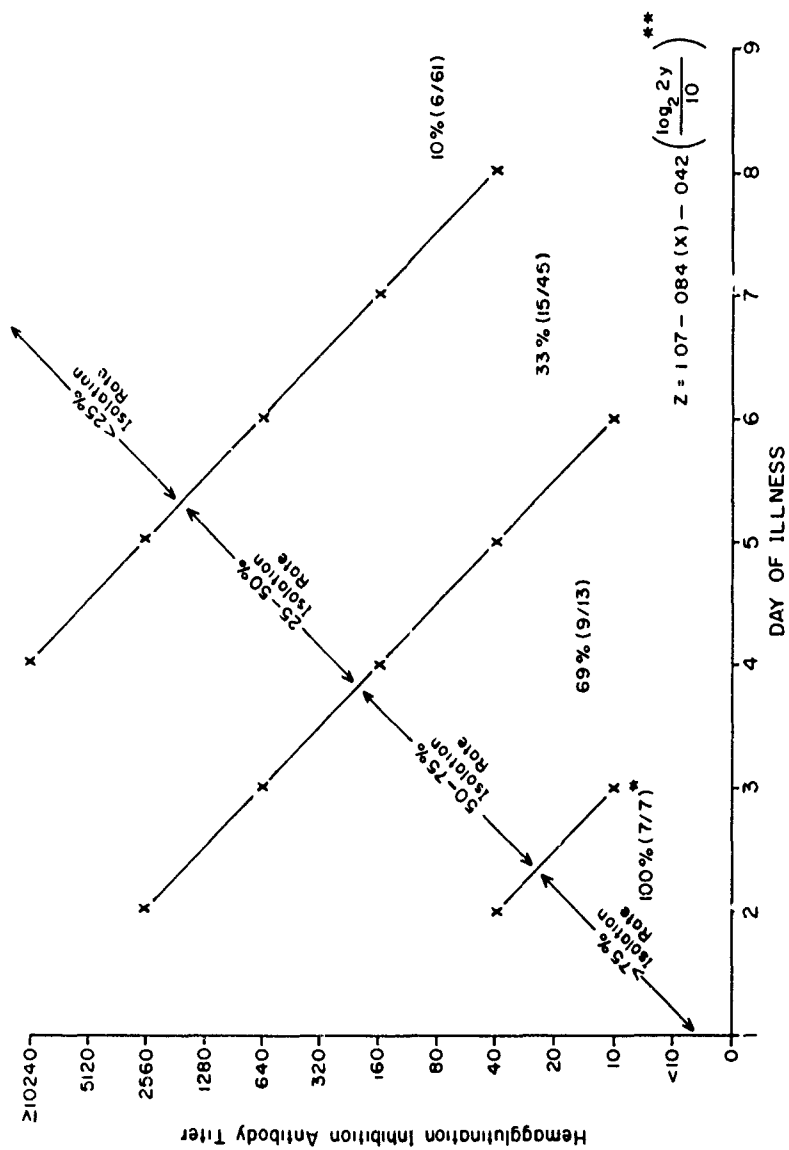


Figure 33 Dengue virus isolation rates in comparison to predicted rates determined by multiple linear regression analysis of actual virus isolation data in relation to the day of disease and the hemagglutination inhibition antibody titers of dengue patients

* actual virus isolation rate

** Z = probability of isolating virus

x = day of illness

y = hemagglutination inhibition antibody titer

The distribution of dengue virus isolations according to age and sex of patients is presented in Table 53. Overall, the rate was slightly higher for males (31%) than for females (27%). The rate of virus isolation varied for different age groups and tended to be higher for patients seven years of age and older. The apparent increase in virus isolation rates for older children mentioned above suggested that they reported to the hospital earlier than younger children infected with dengue viruses. An analysis of the data showed the mean reporting day to be approximately the same in each age group, but the range in the reporting period indicated that the older children did (7-8 and 11-12 age groups) tend to seek medical attention sooner than younger children.

The rate of isolation of dengue viruses in relation to the day of disease and the antibody titer of dengue patients is shown in Figure 30. Virus was isolated from 100% of the patients during days 2 and 3 of illness. Subsequently rate declined significantly ($r = -0.958$, $p = 0.001$) until day 8 when virus could no longer be isolated from patients (Figure 31). The rate of virus isolation for patient with antibody titers that ranged from less than 10 to 160 was 74% (20/27) as compared to a 17% (17/99) rate for patients with antibody titers of 320 or greater. The association of a decrease in virus isolation with an increase in antibody titers of patients was significant ($r = 0.843$, $p = 0.001$) (Figure 32). Multiple linear regression analysis of the data showing the relation of virus isolation to both the day of illness and the HI antibody titer of dengue patients is presented in Figure 33. On the basis of these results, the probability of obtaining dengue virus isolations from patients can be predicted with a high degree of certainty. Employment of these findings in future studies will save both technical input and time, as well as overall cost required to perform the assays.

This is a final report.

20. Isolation of Dengue Viruses from Plasma and Cellular Components of Blood

OBJECTIVE: To determine the relative frequency of isolation of dengue viruses from plasma and cellular components of the blood of dengue patients.

BACKGROUND: Studies of the pathogenesises of dengue hemorrhagic fever (DHF) have implicated human peripheral blood leukocytes (PBL), particularly the phagocytic monocyte, as the primary site

of dengue virus replication. Evidence that supports this proposition includes the association of dengue viruses and/or dengue virus antigens with PBL of the blood of dengue patients and of experimentally infected monkeys (116, 117, 118, 119, 120). In vitro studies have shown that dengue viruses replicate in both monkey and human PBL cultures (121, 122). In additions cultured human B type lymphoblastoid cell lines and macrophages have been shown to support replication of dengue-2 virus (122, 123, 124).

However, attempts to infect polymorphonuclear (PMN) cells, T type human lymphoblastoid cell lines, and T lymphocytes, have not been successful (121, 122). Despite these findings, direct association of dengue viruses with PBL during human dengue virus infection remains to be proven.

METHODS: Heparinized blood was obtained from dengue patients as described previously. Blood specimens were centrifuged at 2000 rpm for 15 minutes and plasma was stored at -20°C and at -70°C for serological and virological studies.

The dextran sedimentation technique employed in a preliminary comparative study with the Isopaque Ficoll (IP-Ficoll) flotation technique for isolating leukocytes was performed as described previously (120).

The IP-Ficoll flotation technique (IP-Ficoll) for separating cellular fractions of the blood of dengue patients was employed as shown in Figure 34. Each cellular fraction was mixed with 4.0 ml of RPMI 1640 medium and carefully layered over IP-Ficoll medium. After centrifugation at 1800 rpms for 30 minutes at 4°C, the top layer of RPMI 1640 medium was carefully removed with a pasteur pipette. The mononuclear cell and platelet fraction of the interface region was transferred to a 15 ml conical centrifuge tube. Cells were then suspended in 5.0 ml of RPMI 1640 medium and centrifuged at 500 rpm for 10 minutes.

The supernatant was transferred to a 15 ml conical centrifuge tube and the pelleted mononuclear cells were resuspended and centrifuged again as above. Supernatants were pooled and centrifuged at 2000 rpm for 10 minutes at 4°C and the pelleted platelets were resuspended in 2.0 ml of RPMI 1640 and a 1.0 ml and a 0.2 ml aliquot was stored at -70°C. Sedimented mononuclear cells were suspended in 2.5 to 3.0 ml of RPMI 1640 medium, with 10% heat inactivated fetal calf serum (FCS). Cell concentration was adjusted to approximately 1×10^6 cells per ml and a 1.0 ml and a 0.2 ml aliquot was stored at -70°C.

Adherent and non-adherent cells were obtained from the remaining portion of mononuclear cell fractions on the basis of glass adherence properties of monocytes. Each mononuclear cell-RPMI 1640 medium fraction (0.5 ml) was transferred to 1 oz glass bottles and placed at 37°C for three hours to allow monocytes to adhere to glass. The non-adherent cell fraction that remained suspended in the RPMI 1640 medium was transferred to 1 oz bottles. Adherent cells were rinsed 2 to 3 times with RPMI 1640 medium. Four ml of a LLC-Mk2 cell suspension, 2×10^6 cells/ml, was then added to each bottle that contained adherent and/or nonadherent cells. After 24 hours incubation at 37°C, 2.5 ml of a standard agar overlay was added to each bottle of cells. A similar volume of a second agar overlay that contained a 1:10,000 dilution of neutral red was added on day 6 post inoculation. Plaque forming units (PFU) were counted and recorded on the following day.

Granulocytes and erythrocytes that comprised the bottom layer of the IP-Ficoll medium were suspended in two volumes of 2.4% dextran (T-250), Hank's balanced salt solution (HBSS) and 0.15% EDTA. The mixture was left at 4°C for 45 minutes to one hour, and the supernatant that contained the granulocytes was transferred to a 15 ml conical centrifuge tube. Supernatant was centrifuged for 10 minutes at 1000 rpm and the pelleted granulocytes were resuspended in HBSS plus 0.02% EDTA. This step was repeated and granulocytes were resuspended in 3.0 ml of RPMI 1640 medium, with 10% heat inactivated FSC, and 1.0 ml and 0.02 ml aliquots were stored at -70°C.

Plasma, platelets, whole leukocyte and whole mononuclear cell, and polymorphonuclear cell fractions were assayed for virus at 32°C by the mosquito inoculation technique employing Toxorhynchites spoendens (125). The direct fluorescent antibody technique was used to test mosquito heads for virus (125). Corresponding thorax-abdomen portions of mosquitoes, and aliquots of the above plasma, platelets etc. fractions were tested for virus at 35°C in LLC-Mk2 cells by direct and delayed plaque assay (126). The volume of inoculum per cell culture was 0.3 ml and 0.85 ul per mosquito. Viruses were identified by plaque reduction neutralizing tests employing monospecific dengue virus types 1, 2, 3 and 4 antisera prepared in rhesus monkeys. A differential cell count was performed on smears of whole leukocytes, mononuclear, non-adherent, polymorphonuclear and platelets fractions using Wright's stain.

Dengue virus types 1, 2, 3, and 4 were used in plaque reduction neutralization tests (PRNT) to determine antibody titers for dengue patients. Plasma were heat treated at 56°C for 30 minutes. Mixtures of equal volumes of four fold dilutions of plasma and approximately 30 to 60 PFUs of each virus were incubated at 37°C for 90 minutes. Medium free monolayers of LLC-Mk2 cells propagated in 24 well plastic plates were inoculated with virus plasma mixtures, 0.1 ml per each of 2 cultures. After incubation at 37°C for 90 minutes, cell monolayers were rinsed with HBSS and overlaid with 0.5 ml of a standard agar overlay. The remaining procedures for plaque assay were similar to those described for adherent and nonadherent cells. Cell cultures were incubated at 35°C during the assay period. Fifty percent plaque reduction neutralization titers were estimated by probit analysis.

Severity of illness of dengue patients was graded according to previously established criteria (127).

RESULTS: The results of a comparative study to evaluate the dextran sedimentation and the IP-Ficoll flotation technique for fractionating cellular components of the blood of dengue patient for virus isolation studies are presented in Table 54. Dengue viruses were isolated from 9 of 27 leukocyte fractions obtained from blood of 27 patients by both leukocyte fractionating techniques. Although the number of virus isolations from leukocyte fractions obtained by the 2 techniques was comparable, viruses were not always isolated from the same leukocyte fractions. Possibly, this was associated with the difference in the relative proportions of the types of cells in fractions obtained by the 2 techniques. As shown in Table 55 the predominate cell type in the fractions obtained by the IP-Ficoll technique was mononuclear cells (93%) whereas fractions associated with the dextran sedimentation technique were comprised of mononuclear (59%) and polymorphonuclear.

The relative proportion of 37 dengue virus isolations associated with one or more of the different types of fractions of the blood of dengue patients is presented in Table 56. Dengue viruses were associated with all types of blood fractions but most frequently with mononuclear cell fractions. Although the data was based on an unequal number of fractions assayed per blood specimen, similar results were obtained by analysing the data for 26 patients for whom all types of fractions were assayed for virus (Table 57).

As shown in Table 58 the adherent cell portion of the mononuclear fraction was responsible for the majority of the additional dengue

Table 54 Dengue Virus Isolations from Leukocyte Fractions
Prepared by the Dextran Sedimentation and the
Isopaque Ficoll Flotation Techniques.

No. of Patients studied ¹	Virus Isolations					Total
	IP-Ficoll	DS	Both ²	IP-Ficoll Only	DS Only	
27	06	07	04	02	03	03

1. All 27 patients studied by both techniques.
2. Virus isolated from the same leukocyte suspension by both technique.

Table 55 Percentage of Leukocytes and Number of Platelets in Cell Fractions Assayed for Dengue Viruses.

Cell Fractions	No. of Fractions	Differential cell count				Platelets
		Polymorpho- Nuclear	Lymphocytes	Monocytes	Atypical Lymphocytes	
Mononuclear	28	1.0%	79.0%	12.0%	8.0%	1.6 ¹
Nonadherent cells	18	3.0	56.0	1.0	40.0	0.5
Polymorphonuclear	25	84.0	15.0	1.0	0.4	1.7
Leukocytes ²	17	41.0	41.0	4.0	14.0	0.3

1. Average count per high powered field
2. Obtained by dextran sedimentation, others obtained by combination of IP-Ficoll, dextran sedimentation and differential centrifugation.

Table 56 Dengue Virus Isolations from Plasma and Cellular Fractions Based on an Unequal Number of Fractions Assayed Per Blood Specimens.

Blood Fractions	No. of Fractions	Total virus Isolation	Virus isolation Alone	% of Total
Plasma	37	25	00	00.0
Mononuclear cells	36	29	05	71.4
Polymorphonuclear cells	27	14	01	14.3
Platelets	27	12	00	00.0
Adherent cells	28	22	01	14.3
Nonadherent cells	28	17	00	00.0
Total		30	07	100.0

Table 57 Dengue Virus Isolations from Blood Fractions of Each Patients Ranked in Decending Order According to the Number of Patients with all Fractions Positive to the Number of Patients with only One Virus Positive Fraction.

Number of Patients (N=26)	Number of Fractions Positive/no. of Fractions	Blood Fractions				
		Plasma	Mono-nuclear	Polymorpho-nuclear	Platelets	Non-adherent
5	6/6	5	5	5	5	5
5	5/6	5	5	4	5	2
4	4/6	4	4	1	0	3
5	3/6	2	2	2	1	4
3	2/6	1	2	0	0	1
4	1/6	0	2	1	0	0
TOTAL		17	20	13	11	15

Table 58 Distribution of Dengue Virus Isolations Among the Adherent and Nonadherent Leukocytes Fractions of Dengue Patients.

Cell Fractions	No. Virus Isolations	Percent of Total Isolates
Adherent cells alone	07	29
Nonadherent cells alone	02	08
Both fractions	15	63
	24	100

virus isolations. Of the blood fractions other than leukocytes, dengue viruses were associated more frequently with plasma than with platelet fractions (Table 59).

According to results of infectious center assay of adherent and nonadherent cell fractions, both monocytes and lymphocytes appeared to support dengue virus replication (Table 60). Of the culture for which infectious centers could be enumerated, the average yield of virus for the 2 types of cell fractions was comparable; however, more infectious centers than could be counted was associated more frequently with the adherent cell fractions. No apparent relation was noted between the number of infectious centers and the grade of disease or the day of disease of the patients.

The distribution of dengue virus isolations among the different blood fractions of dengue patients by day of disease is presented in Table 61. Overall, the pattern of the rate of virus isolation for the blood fractions was similar as indicated by a rapid decrease in rates subsequent to day 3 of disease. Although the rates for different fractions did not appear to differ significantly, the pattern of decrease for plasma and platelet fractions was more similar and faster than that of the leukocyte, especially the adherent cell fractions.

The relation of antibody titer to the isolation of dengue viruses from plasma and leukocyte fractions of the blood of dengue patients is presented in Table 62. Dengue viruses were isolated from each type of fraction of patients with HI and plasma neutralizing antibody titers that ranged from <10 to $>10,240$ and from undetectable to >640 , respectively. Significantly more virus isolation, however, were obtained from leukocytes than from plasma fractions of patients with high neutralizing antibody titers ($\chi^2 = 5.619$, $p = .018$). A similar relationship was observed between HI antibody titers and the pattern of isolation of dengue viruses from the 2 types of blood fractions.

The distribution of dengue virus isolations among the different blood fractions in relation to the immune response of dengue patients is presented in Table 63. No striking pattern was noted in the distribution of virus isolations among the different fractions of blood of patients who serconverted. In contrast, a marked reduction occurred in the rate of isolation of dengue viruses from platelet fractions of the patients with high fixed antibody titers.

Table 59 Isolations of Dengue Viruses from Blood Fractions
Other than Leukocyte Fractions.

Blood Fractions	No. Virus Isolations
Plasma alone	06
Platelets alone	00
Both fractions	12

Twenty-seven of each fraction assayed for virus.

Table 60 Range and Average Number of Dengue Virus Infectious
Center for Adherent and Non-adherent Cells.

Mononuclear Cell Fractions	Cellular Fractions ¹	<u>Plaque forming units</u>		
		Range	Average	TNTC ²
Adherent Cells	22	1-64(14) ³	15.7	8
Non-adherent Cells	17	5-52(14)	17.9	3

1. Includes fractions that yielded one or more PFU.
2. Too numerous to count
3. () Number of fractions from which 1 to 64 plaque forming units were obtained.

Table 61 Dengue Virus Isolations from Plasma and Cellular Components of the Blood of Dengue Patients by Day of Disease.

Day of Disease	Blood Fractions					
	Plasma(%)	Mononuclear Cells(%)	Polymorphonuclear Cells(%)	Platelets(%)	Adherent Cells(%)	Non-adherent Cells(%)
2	4/05(80) ¹	5/05(100)	3/05(60)	4/05(80)	3/05(60)	4/05(80)
3	2/02(100)	2/02(100)	2/02(100)	2/02(100)	2/02(100)	2/02(00)
4	8/21(38)	9/21(43)	3/12(25)	3/11(27)	4/10(40)	3/10(30)
5	9/38(24)	8/36(22)	3/25(12)	2/19(11)	9/25(36)	7/25(28)
6	2/29(07)	5/24(21)	3/13(23)	1/12(08)	3/13(23)	3/13(23)
7	0/18(00)	0/16(00)	0/11(00)	0/10(00)	1/07(14)	0/07(00)
8	0/06(00)	0/06(00)	0/02(00)	0/02(00)	0/03(00)	0/03(00)
9	0/04(00)	0/03(00)	0/02(00)	0/03(00)	0/03(00)	0/03(00)
10						
11	0/01(00)	NT ²	NT	NT	NT	NT
Total	25/124(20)	29/113(26)	14/72(19)	12/64(19)	22/68(32)	19/68(28)

1. Number virus isolations/total number of blood fractions assayed
2. Not tested.

Table 62 Dengue Virus Isolations from Plasma and Leukocyte Fractions of Dengue Patients in Relation to the Day of Disease and Homologous Hemagglutination Inhibition and Plaque Reduction Neutralization Antibody Titers.

Patient Number	Fractions		Antibody titer	
	Plasma	Leukocytes	HAI	PRNT
D78-009	0 ¹	+ ²	320	640
D78-014	0	+	2560	640
D78-055	0	+	2560	640
D78-097	0	+	10240	640
D78-130	0	+	5120	640
D78-091 ³	0	+	10	25
D78-052	+	+	1280	096
D78-054	+	+	80	160
D78-059	+	+	2560	640
D78-044	+	+	20	32
D78-050	+	+	10	00
D78-051	+	+	40	40
D78-078	+	+	160	190
D78-080	+	+	2560	640
D78-099	+	+	10	35
D78-108	+	+	80	180
D78-112	+	+	10	00
D78-117	+	+	10	130
D78-133	+	+	10	350
D78-145	+	+	160	640
D78-159	+	+	640	640

1. Virus not isolated
2. Virus Isolated
3. Primary infection

TABLE 63 Dengue virus isolations from plasma and cellular fractions of the blood of dengue patients according to the immunological response

Patient Number	Day of Disease	Grade of Illness	Blood Fractions					HA.I. Antibody Titers ¹	
			Plasma	Mono-Nuclear	Polymorpho-Nuclear	Platelets	Adherent Non-Adherent		
D78-091	04	PUO	0	+	0	0	0	0	$\leq 10^2$
D78-108	04	PUO	+	+	0	0	+	+	80
D78-114	02	PUO	+	+	+	+	+	+	$\leq 10^3$
D78-117	03	PUO	+	+	+	+	+	0	10
D78-118	02	PUO	0	+	0	0	0	0	$\leq 10^3$
D78-132	04	PUO	+	+	0	0	+	+	160^3
D78-133	02	PUO	+	+	+	+	0	+	≤ 10
D78-157	05	PUO	+	+	NT ⁴	NT	NT	NT	$\leq 10^3$
D78-009	04	III	NT	+	NT	NT	NT	NT	320
D78-025	04	III	+	+	NT	NT	NT	NT	20
D78-044	04	II	+	+	+	+	+	+	20
D78-050	03	III	+	+	+	+	+	0	≤ 10
D78-051	05	III	+	+	0	0	+	+	40
D78-054	05	II-III	+	0	0	0	+	0	80
D78-069	02	II	+	+	0	+	+	+	10^3
D78-078	05	III	+	+	0	+	0	0	160
D78-099	06	III	+	+	+	+	+	+	≤ 10
D78-112	05	II-III	+	+	+	+	+	+	≤ 10
D78-135	02	II	+	+	+	+	+	+	10
D78-145	05	III-IV	+	+	NT	NT	+	+	160
D78-168	05	II	+	+	NT	NT	NT	NT	80
D78-014	04	III	NT	+	NT	NT	NT	NT	2560
D78-017	04	II	+	+	NT	NT	NT	NT	1280
D78-026	06	II	0	+	NT ²	NT	NT	NT	5120
D78-042	04	III	+	+	+	+	+	+	5120
D78-048	05	III	0	0	+	0	+	+	640
D78-052	05	II-III	+	NT	NT	NT	+	+	1280
D78-055	05	III	0	0	+	0	+	+	2560
D78-059	05	III	+	0	0	0	+	+	2560
D78-074	06	III	0	+	0	0	+	+	5120
D78-077	05	III	0	+	0	0	+	0	2560
D78-080	06	III	+	+	+	0	+	0	2560
D78-084	07	I	0	0	0	0	+	0	2560
D78-097	06	II	0	0	+	0	0	0	>10240
D78-130	06	II	0	+	0	0	0	+	5120
D78-136	04	III	+	+	+	+	+	+	1280
D78-159	04	III	+	+	NT	NT	NT	NT	640

1. Reciprocal homologous HA.I. antibody titers

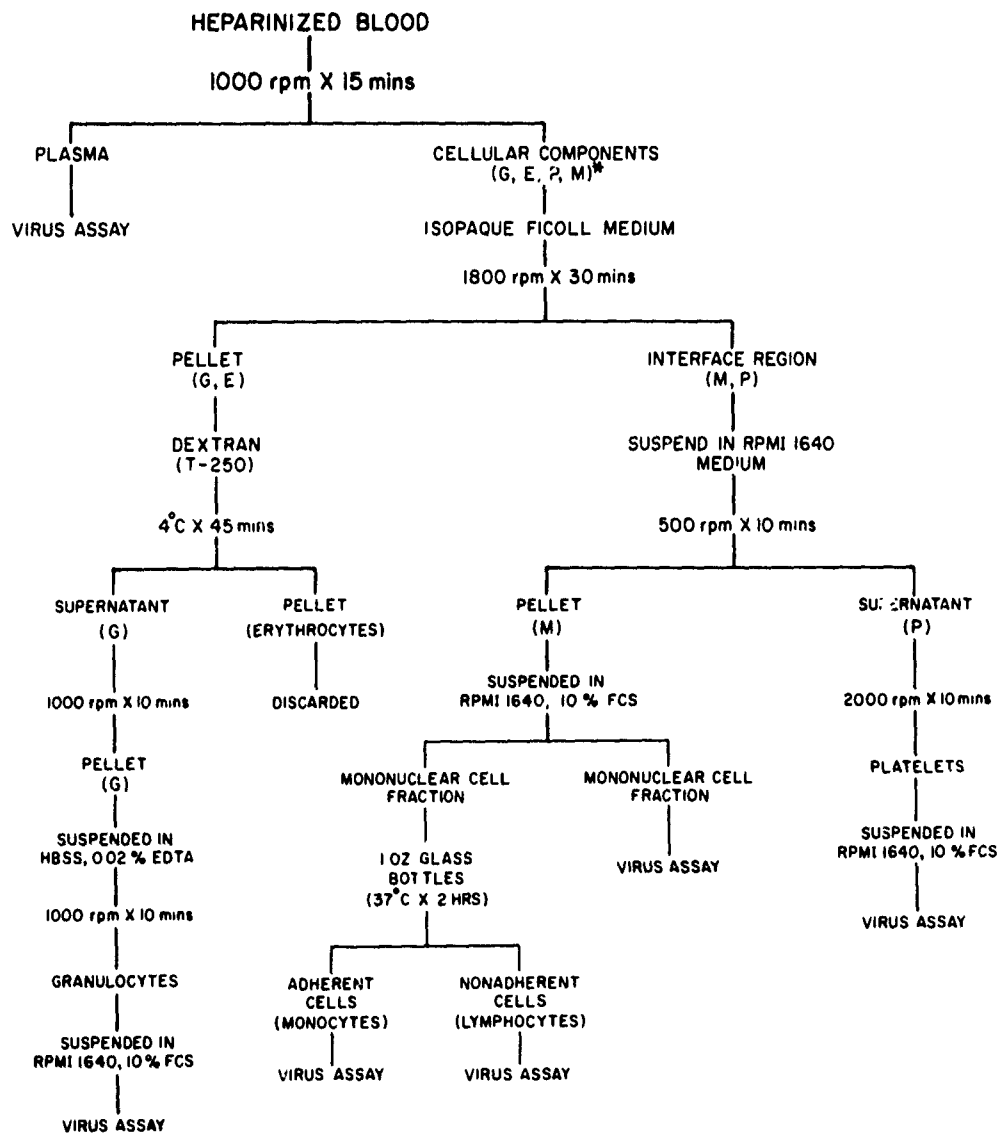
2. Primary immune response, all others secondary immune response except for unpaired plasma specimens

3. Unpaired plasma specimens

4. Not tested

5. High fixed antibody titer

Figure 34 Separation and isolation of plasma and cellular components of the blood of dengue patients



* G = GRANULOCYTES, E = ERYTHROCYTES, P = PLATELETS, M = MONONUCLEAR

The pattern of virus isolation did not appear to be related to the severity of disease, but the decrease in the rate of isolation of dengue viruses from platelets fraction tended to be associated with the late phase of illness of the patients.

This is a final report.

21. Comparison of the Mosquito Inoculation Technique With Cell Culture Techniques for Isolating Dengue Viruses From Dengue Patients

OBJECTIVES: To compare Toxorhynchites splendens to LLC-Mk2 cells for isolating dengue viruses from plasma and cellular components of the blood of dengue patients.

BACKGROUND: Dengue virus isolation rates from dengue patients have been shown to vary according to the assay technique and the type of specimens assayed for virus. Recently, the isolation rate reported for the mosquito inoculation assay (128-130) has been substantially higher than the rates obtained by cell culture techniques (131-133, 23, 134, 135). In preliminary studies, the rate of isolation of dengue viruses from peripheral blood leukocytes was over three times greater than that from plasma of dengue patients (23). This difference, however, may have been due to the use of a different assay technique for the two types of specimens. The present report describes the results of investigations designed to evaluate and compare the mosquito inoculation assay to cell culture for isolating dengue viruses, and to identify the component(s) of the blood of dengue patients that yielded the maximum number of virus isolations.

METHODS: Plasma and platelet and leukocyte fractions were obtained from blood of dengue patients in the Children's Hospital during 1978 (this Annual Progress Report). Toxorhynchites splendens were from a laboratory colony maintained as described previously (23). LLC-Mk2 cell cultures were propagated with medium 199, with 15% calf serum, in 1 oz glass bottles.

Figure 35 shows the scheme used to assay fractions of the blood of dengue patients for virus. Blood fractions were assayed for virus by the mosquito inoculation technique employing Tx. splendens (this Annual Progress Report), and in LLC-Mk2 cells by the direct and delayed plaque assay (136) and the infectious center assay (this Annual Progress Report). Eight or more Tx. splendens were inoculated intrathoracically with each of the undiluted cellular specimens and a 1:5 dilution of plasma, 0.85 ul per mosquito.

After 14 days incubation at 32°C, mosquitoes were stored at -70°C. Head squashes of individual mosquitoes were examined for virus by the direct fluorescent antibody technique (this Annual Progress Report) and corresponding thorax-abdomens were tested for virus by the direct and delayed plaque assay. The amount of inoculum was 0.3 ml per cell culture. Viruses were identified by the plaque reduction neutralization test using monospecific dengue virus types 1, 2, 3, and 4 antisera prepared in rhesus monkeys. Cell culture assays were performed at 35°C.

RESULTS: Isolation rates for dengue viruses from plasma and cellular components of the blood of dengue patients, by the mosquito inoculation and by the direct and delayed plaque assay, are shown in Table 64. An analysis of the variation in rates obtained by the mosquito inoculation and the cell culture assays for the different types of blood fractions is presented in Table 65. A total of 17 virus isolations were obtained from the same plasma fractions by both the mosquito inoculation and the cell culture assays. An additional seven plasma fractions yielded virus isolations by the mosquito inoculation technique, while one additional virus isolation was obtained by the cell culture technique.

In contrast, the total number of isolations obtained by the mosquito inoculation and the cell culture assays from each of the cellular fractions were comparable. However, there was considerable variation in the two techniques for detecting virus in the same cellular fractions. Forty-six percent of the virus isolations from mononuclear cell fractions, 43% of the isolations from polymorphonuclear cells and 33% of the isolations from platelet fractions were not detected by both techniques. Virus isolations obtained from blood fractions only by cell culture assay, were by the delayed plaque assay technique. Thus, the mosquito inoculation technique was more effective than the direct plaque assay, but comparable to the delayed plaque assay for obtaining dengue viruses from cellular fractions of the blood of dengue patients.

The variation in the virus isolation rates obtained by the mosquito inoculation and the cell culture assays for the different blood fractions was analysed according to the HI antibody and the neutralizing antibody titers of the patients. Isolations of dengue viruses only from plasma by the mosquito inoculation technique appeared to be related to the antibody titers, especially HI antibody (Table 66). The geometric mean antibody titer for patients from whom virus isolations were obtained from the same plasma fractions by both techniques was 1:33 while the titer was

1:352 for patients from whom viruses were isolated only by the mosquito inoculation technique. A similar relationship was noted between HI antibody titers and the isolation of dengue viruses from patients. In contrast, the variation in the pattern of virus isolation from platelets and polymorphonuclear fractions by the two techniques was not related to antibody titers of the patients (Table 67 and 68). Isolation of dengue viruses from mononuclear cell fractions appeared to be related to antibody titer of the patient, but the pattern in regard to the techniques was the opposite of that observed for plasma fractions (Table 69). Four virus isolations were obtained only by the mosquito inoculation technique from mononuclear cell fractions of patients with an HI antibody less than 10 (in most cases), while 8 isolations were obtained only by the delayed plaque assay, from patients primarily with high fixed antibody titers.

Sixty-nine adherent and nonadherent cell fractions were tested for virus by the infectious center assay in LLC-Mk2 cells. The rate of isolation of dengue viruses by the above technique as compared to the mosquito inoculation and the direct and delayed plaque assay is presented in Table 70. Isolation rates for adherent and nonadherent cell fractions were higher than those observed for other fractions assayed by either the mosquito inoculation or the direct and delayed plaque assay. However, the rates were similar to those obtained for mononuclear cell fraction by each of the other techniques. Overall isolation rates for the mononuclear cell fractions assayed by both the mosquito inoculation and the direct and delayed plaque assay were 36%. The isolation rates for the adherent and nonadherent fractions tested by the infectious center assay was 35%. This similarity in the number of virus isolations is not surprising since the adherent and nonadherent cell originated from the mononuclear cell fractions. The only difference in the techniques was the volume of inoculum for the infectious center assay which was approximately twice that used for either of the other techniques. The larger volume of inoculum may have been responsible for the higher isolation rates associated with the infectious center assay as compared to each of the other techniques.

Table 71 shows the F.A. assay results for head smears of Tx. splendens in comparison to the results of direct and delayed plaque assays of corresponding thorax-abdomen suspensions. An exceptionally high correlation was observed between F.A. virus positive mosquito heads and the recovery of virus from corresponding thorax-abdomen suspensions by the direct and delayed plaque assay. Isolation of dengue viruses from thorax-abdomen suspensions with no visible fluorescence in corresponding mosquito heads, however, was observed more frequently. Overall, for the

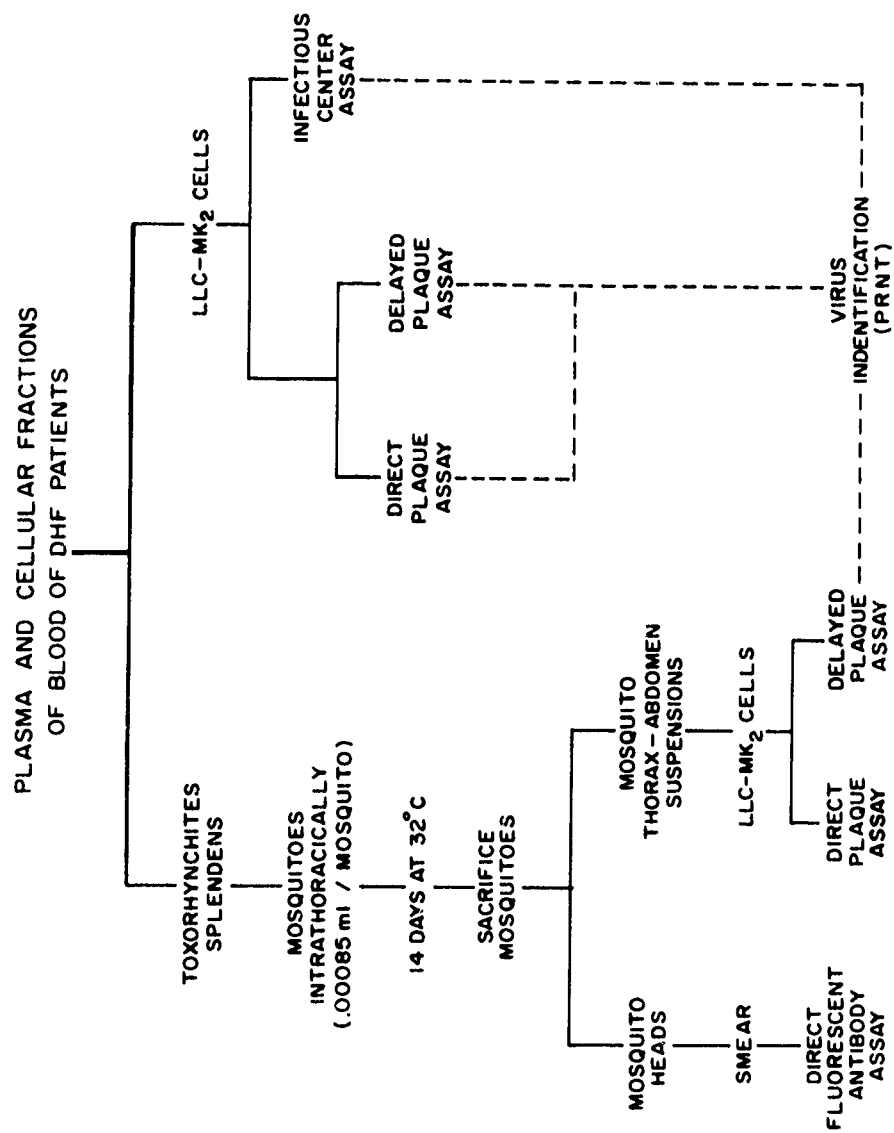


Figure 3. Procedure used to assay fractions of the blood of dengue patients for virus

Table 64 Dengue Virus Isolations from Plasma and Cellular Components of the Blood of Dengue Patients.

Blood Fractions	Virus Assay Technique				Total ¹
	Plaque Assay (%)		Tx. splendens		
Plasma	18/116	(16) ²	24/116	(21)	25/116(22)
Mononuclear Cells	21/103	(19)	21/103	(20)	27/103(26)
Polymorphonuclear Cells	11/71	(15)	11/71	(15)	14/71(20)
Platelets	09/64	(14)	10/64	(16)	12/64(19)

1. Number of virus isolations obtained by both assay techniques.
2. Number of virus isolations/number of fractions tested.

Table 65 Summary of Dengue Virus Isolations Obtained by Different Assay Techniques from Blood Fractions of Dengue Patients.

Blood Fractions	Tx. splendens and LLC-Mk2		Tx. splendens Only		LLC-Mk2 Only		Direct and Delayed ²		Total
Plasma	17	07 (87.5)	01 (12.5)	15 (83)	03 (17)			25	
Mononuclear Cell	15	05 (45)	06 (55)	11 (55)	09 (45)			27	
Polymorphonuclear Cell	08	03 (50)	03 (50)	05 (45.5)	06 (54.5)			14	
Platelets	07	03 (60)	02 (40)	04 (44)	05 (56)			12	
Total		18 (60)	12 (40)	35 (60)	23 (40)				

1. Virus isolations obtained only by the delayed plaque assay
2. Virus isolations obtained by both direct and delayed plaque assay in LLC-Mk2 cells
3. One virus isolation obtained by mosquito assay from specimen that was not tested by cell culture assay

Table 66 Isolation of Dengue Viruses from Plasma of Dengue Patients by the Mosquito Inoculation Technique and by the Direct and Delayed Plaque Assay in Relation to Hemagglutination Inhibition Antibody and Neutralizing Antibody Titers.

Patients Number	Day of Disease	Technique		Antibody Titer	
		Mosquito Assay	Plaque Assay LLC-Mk2	HI ¹	PRNT ²
D78-017	4	+ ³	0 ⁴	640	ND
D78-042	4	+	0	5120	ND
D78-054	5	+	0	80	160
D78-059	5	+	0	2560	640
D78-108	4	+	0	80	180
D78-145	5	+	0	160	640
D78-168	5	+	0	80	ND
Ave. 4.6				GM 352 ⁵	
D78-080	6	0	+	2560	640
D78-025	4	+	+	20	ND
D78-044	4	+	+	20	32
D78-050	3	+	+	10	00
D78-051	5	+	+	320	040
D78-052	5	+	+	1280	096
D78-069	2	+	+	10	ND
D78-078	5	+	+	160	190
D78-099	6	+	+	10	35
D78-112	5	+	+	10	00
D78-114	2	+	+	10	ND
D78-117	3	+	+	10	130
D78-132	4	+	+	160	ND
D78-133	2	+	+	10	350
D78-135	2	+	+	10	ND
D78-136	4	+	+	1280	ND
D78-157	5	+	+	10	ND
D78-159	4	+	+	640	640
Ave. 3.8				GM 1:33 ⁵	
Total		24	18		

1. Homologous reciprocal hemagglutination inhibition antibody titer
2. Homologous reciprocal plaque reduction neutralization antibody titers
3. Virus negative
4. Virus positive
5. Geometric mean titer

Table 67 Isolation of Dengue Viruses from Platelet Fractions of Dengue Patients by the Mosquito Inoculation Isolation Technique and the Direct and Delayed plaque Assay in Relation to the Hemagglutination Inhibition Antibody and Neutralizing Antibody Titers.

Patient Number	Day of Disease	Technique			Antibody Titers	
		Mosquito Assay	LLC-Mk2 Assay	Plaque Assay	HI	PRNT
D78-078	5	+ ¹	0 ²		160	190
D78-117	5	+	0		10	000
D78-044	4	0	+		020	032
D78-136	4	0	+		1280	ND
D78-117	3	+	0		10	130
D78-042	6	+	+		5120	ND
D78-050	3	+	+		10	00
D78-069	2	+	+		10	ND
D78-099	6	+	+		10	035
D78-114	2	+	+		10	ND
D78-133	2	+	+		10	350
D78-135	2	+	+		10	ND
Total		10	09			

1. Virus positive
2. Virus negative

Table 68 Isolation of Dengue Viruses from Polymorphonuclear Cell Fractions of Dengue Patients by the Mosquito Inoculation and Direct and Delayed Plaque Assay in Relation to Hemagglutination Inhibition Antibody and Neutralizing Antibody Titers.

Patient Number	Day of Disease	Techniques		Antibody Titers	
		Mosquito Assay	Plaque Assay	HI	PRNT
D78-050	3	+ ¹	0 ²	10	00
D78-055	5	+	0	2560	ND
D78-097	6	+	0	10240	ND
D78-042	6	0	+	5120	ND
D78-044	4	0	+	020	32
D78-048	5	0	+	640	ND
D78-080	6	+	+	2560	640
D78-099	6	+	+	10	035
D78-112	5	+	+	10	000
D78-114	2	+	+	10	ND
D78-117	3	+	+	10	130
D78-133	2	+	+	10	350
D78-135	2	+	+	10	ND
D78-136	4	+	+	1280	ND
Total		11	11		

1. Virus positive

2. Virus negative

Table 69 Isolation of Dengue Viruses from Mononuclear Cell Fractions of Dengue Patients by the Mosquito Inoculation Technique and Cell Culture in Relation to Hemagglutination Inhibition Antibody and Neutralizing Antibody Titers.

Patient Number	Disease	Technique		Antibody Titers	
		Mosquito Assay	Plaque Assay	HI	PRNT
D78-017	4	+ ¹	ND ²	1280	ND
D78-026	6	+	0	5120	ND
D78-050	3	+	0	10	00
D78-069	2	+	0	10	ND
D78-091	5	+	0	10	ND
D78-118	2	+ ³	0	10	ND
D78-009	4	0	+	320	ND
D78-014	4	0	+	2560	ND
D78-044	4	0	+	20	32
D78-074	6	0	+	5120	ND
D78-077	5	0	+	2560	640
D78-108	6	0	+	5120	ND
D78-025	4	+	+	1280	ND
D78-051	5	+	+	320	040
D78-078	5	+	+	160	190
D78-099	6	+	+	10	035
D78-112	5	+	+	10	00
D78-114	2	+	+	10	ND
D78-117	3	+	+	10	130
D78-132	4	+	+	160	ND
D78-133	2	+	+	10	350
D78-135	2	+	+	10	ND
D78-136	4	+	+	1280	ND
D78-145	5	+	+	160	ND
D78-157	5	+	+	10	ND
D78-159	4	+	+	640	640
D78-168	5	+	+	80	ND
Total		21	21		

1. Virus positive
2. Not Done
3. Virus negative

Table 70 Summary of Dengue Virus Isolations that were Obtained from Dengue Patients Employing Three Different Virus Assay Techniques.

	Virus Assay Techniques			Total Isolations
	Tx. splendens	Plaque Assay - LLC-Mk2	Infectious Center Assay	
Plasma	18/68(.26) ²	15/69(.22)	-	19/69(27)
Mononuclear Cell	15/59(.26)	16/57(.28)	-	21/59(36)
Polymorphonuclear Cell	11/64(.17)	10/64(.16)	-	14/64(22)
Platelet	9/58(.16)	10/58(.17)	-	11/58(19)
Adherent Cell	-	-	22/69(.32)	
Nonadherent Cell	-	-	17/69(.25)	24/69(35)

1. Virus isolation rates were based on the total number of the same fractions that were assayed by all techniques.

2. Number virus isolations/number of fractions tested (%)

Table 71 Results of Fluorescent Antibody Tests for Mosquito Head Smears, and Direct and Delayed Plaque Assays for Corresponding Thorax-abdomens of the Mosquito Heads.

Blood Fraction	Total virus Isolations	Mosquito Heads	
		Fluorescent Antibody	Thorax-abdomen Plaque Assay
Plasma	24	21 (88)	24
Mononuclear	21	19 (95)	21
Polymorphonuclear	10	09 (90)	10
Platelets	10 ²	09 (90)	09
Total	65	58 (89)	64 (98)

1. Percent of total virus isolations
2. One platelet fraction was virus positive by the F.A. mosquito head smear but the corresponding thorax-abdomen suspension was negative for virus by the direct and delayed plaque assays.

different blood fractions, virus was detected only in thorax-abdomen suspensions for approximately one of every 10 virus isolations. In each instance, the isolate was dengue virus type-2.

22. Evaluation of *Toxorhynchites splendens* (Diptera: Culicidae) as a Bioassay Host for Dengue Viruses

OBJECTIVES: To develop a technique for isolation dengue viruses employing *Toxorhynchites splendens* and the direct fluorescent antibody assay.

BACKGROUND: This is a continuation of an investigation that was initiated during 1977 to develop and evaluate the mosquito assay technique for detecting and isolating dengue viruses (23). Previous studies showed *Aedes aegypti* and *Toxorhynchites splendens* to be equally susceptible to dengue virus infection following intrathoracic inoculation. Additional findings, however, indicated that, of the two species, *T. splendens* was a more suitable host. The mortality rate following inoculation of *T. splendens* was substantially lower than that observed for *Ae. aegypti* and the large size of *T. splendens* as compared to *Ae. aegypti* allowed for a five fold increase in the volume of inoculum. Thus the number of mosquitoes required to be inoculated per specimen was reduced by 50%. In addition, the virus yield per individual mosquito was consistently higher for *T. splendens* than for *Ae. aegypti*. The only disadvantage of using *T. splendens* was the additional time and personnel that were required to mass rear this species as compared to *Ae. aegypti*.

The specific objective of this study was to determine the pattern of replication of different dengue virus serotypes and/or strains and to determine the earliest post-inoculation day that dengue virus antigen could be detected in the heads of mosquitoes by the direct fluorescent antibody technique.

MATERIALS AND METHODS: The dengue virus serotypes and strains employed are presented in Table 72. Eight or more *T. splendens* were inoculated intrathoracically with each virus dilution, 0.85 ul per mosquito. Viruses were diluted in RPMI 1640 medium that was supplemented with heat inactivated fetal calf serum (FCS) to a final concentration of 10%, 200 units of penicillin/ml, and 150 ug of streptomycin/ml. After various time (days) intervals at 32°C, five or more mosquitoes were stored at -70°C for virus assay. Tissue smears of individual mosquito heads were prepared and examined for virus antigens by the direct fluorescent antibody technique.

Table 72 Dengue Viruses Used in Investigations to Evaluate
Toxorhynchites splendens as a Bioassay Host for
these Viruses.

Dengue virus serotype	Origin/Date	Host/No. of passages
Den-1 (001)	Human/1975	Mouse/5*
Den-2 (3379)	Human/1974	Mouse/5
Den-2 (189-18A)	<u>A. aegypti</u> /1978	<u>T. splendens</u> /1
Den-3 (2797)	Human/1977	Mouse/5
Den-3 (2877)	Human/1977	Mouse/5
Den-4 (050)	Human/1977	Mouse/5

* Stock virus prepared from infected suckling mouse brain.

Table 73 Concentrations of Dengue Virus Type-1 in Thorax-abdomen Tagmata of Toxorhynchites splendens Determined by Cell Culture Assay and Detection of Virus Specific Fluorescence in the Heads of the Same Mosquitoes Following Intrathoracic Inoculation.

<u>T. splendens</u>	Days after inoculation					
	3	6	9	12	15	18
Thorax-abdomens	2.5*	4.8	1.8	3.0	1.0	3.0
	3.1	4.3	2.2	2.5	2.5	2.5
	3.5	4.1	1.5	2.0	2.0	1.9
	2.8	4.0	1.6	2.5	2.7	2.5
Heads	0/4**	1/4	4/4	4/4	4/4	4/4
Controls***	0/4	0/4	0/4	0/4	0/4	0/4

* \log_{10} PFU/1.0 ml, dengue-1(001) per individual thorax-abdomen tagmata of T. splendens.

** Number positive/number assayed by head squash.

*** Not inoculated.

Table 74 Concentration of Dengue Virus Type 2 Strains in Thorax-Abdomen Tagmata of Toxorhynchites splendens Determined by Cell Culture Assay and Detection of Virus Specific Fluorescence in the Heads of the Same Mosquitoes Following Intrathoracic Inoculation.

<u>T. splendens</u>	Days after inoculation					
	3	6	9	12	15	18
Thorax-abdomens	1.3	4.8	5.6	3.8	3.5	5.0
	0.5	4.5	5.5	4.8	3.5	4.2
	2.0	5.6	4.8	4.5	3.5	4.2
	2.5	5.3	5.2	3.8	2.8	2.8
Heads	0/4**	0/4	4/4	4/4	4/4	4/4
Controls***	0/4	0/4	0/4	0/4	0/4	0/4

* Log_{10} PFU/1.0 ml, dengue-2 (189-18A) per individual thorax-abdomen tagmata of T. splendens.

** Number positive/number assayed by head squash.

*** Not inoculated.

<u>T. splendens</u>	Days after inoculation					
	3	6	9	12	15	18
Thorax-abdomens	4.5*	5.8	5.5	5.1	4.5	5.1
	4.2	5.7	5.5	4.8	5.1	5.2
	4.0	6.2	5.3	5.5	4.8	5.6
	4.1	6.4	5.5	5.3	5.8	5.1
Heads	0/3**	0/4	4/4	4/4	4/4	4/4
Controls***	0/4	0/4	0/4	0/4	0/4	0/4

* Log_{10} PFU/1.0 ml, dengue-2 (3379) per individual Thorax-abdomen tagmata to T. splendens.

** Number positive/number assayed by head squash.

*** Not inoculated.

Table 75 Concentrations of Dengue Virus Type-3 Strains in Thorax-abdomen Tagmata of Toxorhynchites splendens Determined by Cell Cultures Assay and Detection of Virus Specific Fluorescence in the Head of the Same Mosquitoes Following Intrathoracic Inoculation.

<u>T. splendens</u>	Days after inoculation					
	3	6	9	12	15	18
Thorax-abdomens	0.0 2.0* 2.5 0.5	4.5 4.2 3.9 3.5	1.8 1.8 4.0 2.5	3.8 2.5 3.8 3.3	2.5 3.5 2.5 2.8	2.5 2.5 4.0 2.8
Heads	0/4**	0/4	3/4	3/4	4/4	4/4
Controls***	0/4	0/4	0/4	0/4	0/4	0/4

* Log_{10} PFU/1.0 ml, dengue-3 (2797) per individual thorax-abdomen tagmata of T. splendens.

** Number positive/number assayed by head squash.

*** Not inoculated.

<u>T. splendens</u>	Days after inoculation					
	3	6	9	12	15	18
Thorax-abdomens	3.5* 2.5 2.0 3.0	1.5 2.8 0.7 2.0	4.0 4.5 4.2 4.3	4.0 3.1 3.3 2.5	4.0 3.2 ND** ND	2.5 4.8 2.8 3.8
Heads	0/4***	0/4	0/4	4/4	4/4	4/4
Controls****	0/4	0/4	0/4	0/4	0/4	0/4

* Log_{10} PFU/1.0 ml, dengue-3 (2877) per individual thorax-abdomen tagmata of T. splendens.

** Not done.

*** Number positive/number assayed by head squash.

**** Not inoculated.

Table 76 Concentrations of Dengue Virus Type 4 in Thorax-abdomen Tagmata of Toxorhynchites splendens Determined by Cell Culture Assay and Detection of Virus Species Fluorescence in the Head of the Same Mosquitoes Following Intrathoracic Inoculation.

<u>T. splendens</u>	Days after inoculation					
	3	6	9	12	15	18
Thorax-abdomens	3.8*	4.7	2.3	3.0	2.5	4.1
	4.3	5.1	2.5	4.1	3.8	5.0
	3.5	4.7	2.5	3.8	3.8	4.1
	3.8	4.6	2.6	3.8	3.3	3.5
Heads	0/4**	0/4	2/4	4/4	4/4	4/4
Controls***	0/4	0/4	0/4	0/4	0/4	0/4

* \log_{10} PFU/1.0 ml, dengue-4 (050) per individual thorax-abdomen tagmata of T. splendens.

** Number positive/number assayed by head squash

*** Not inoculated.

Figure 36 Perinuclear Fluorescence in Ganglia Cells of Toxorhynchites splendens Inoculated with Dengue Virus Type 1.

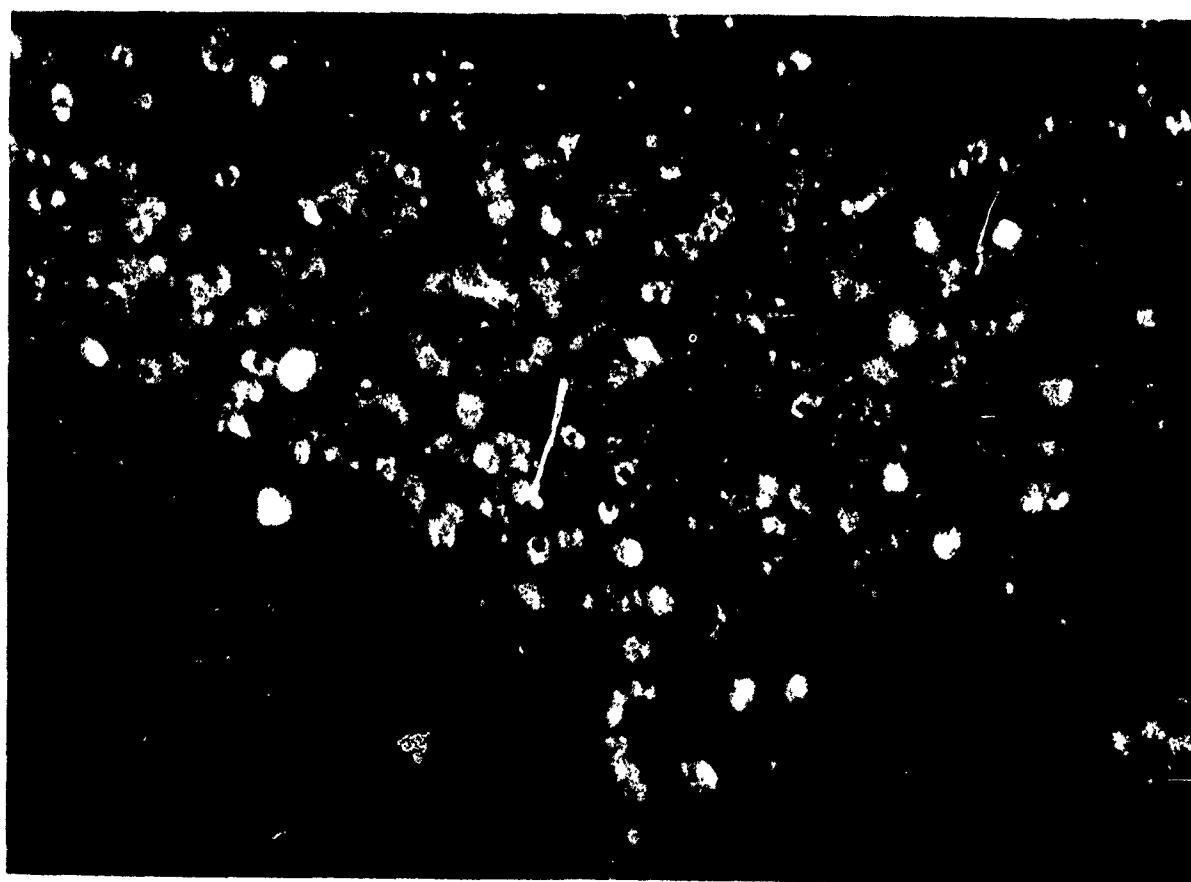


Figure 37 Perinuclear Fluorescence in Ganglia Cells of Toxorhynchites splendens Inoculated with Dengue Virus Type 2.

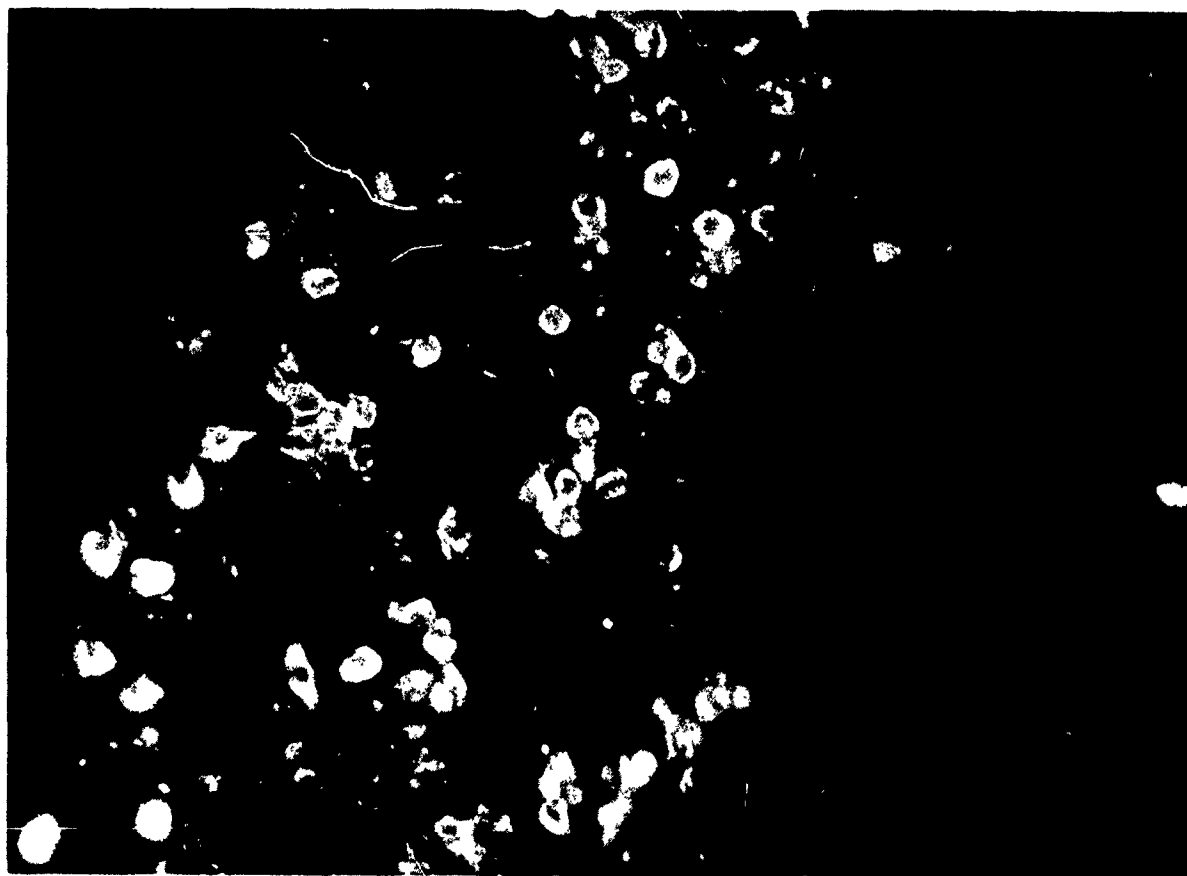


Figure 38 Perinuclear Fluorescence in Ganglia Cells of
Toxorhynchites splendens Inoculated with Dengue
Virus Type 3.

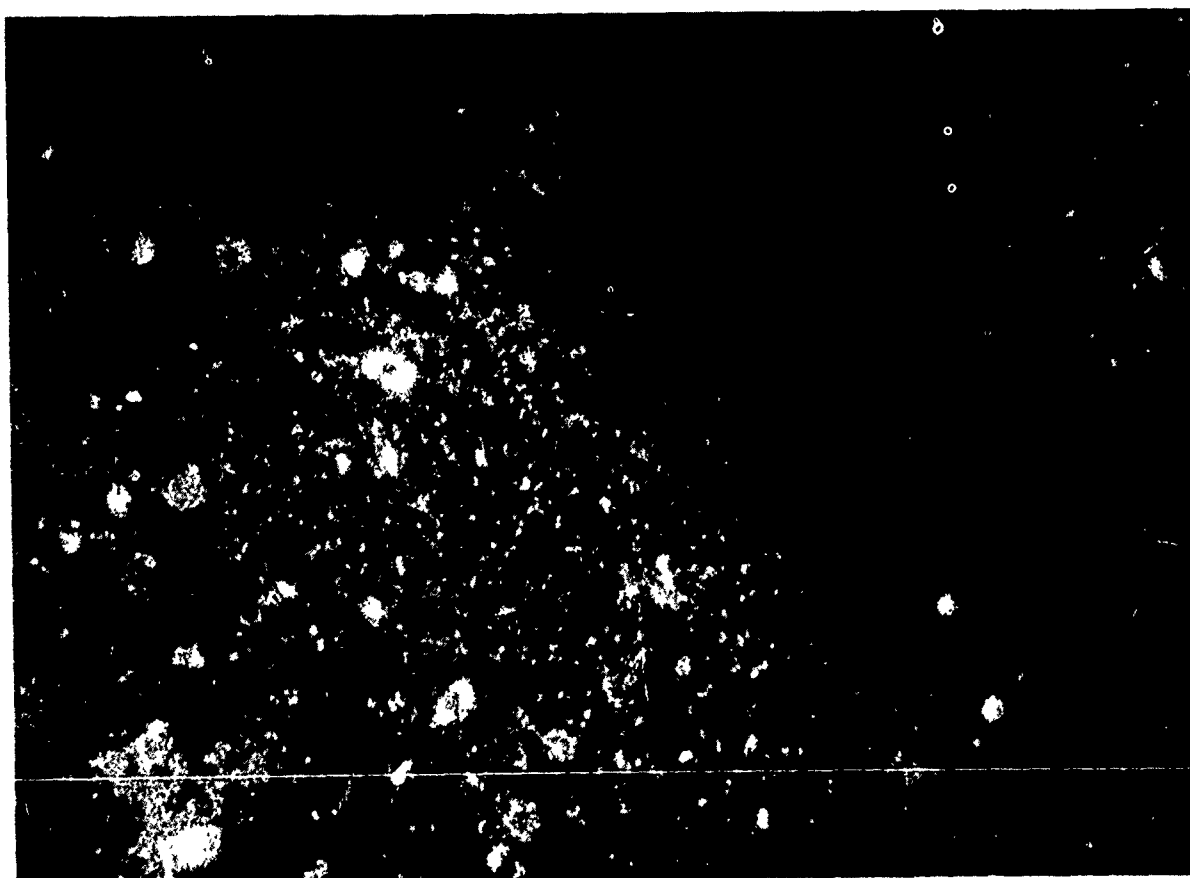


Figure 39 Perinuclear Fluorescence in Ganglia Cells of
Toxorhynchites splendens Inoculated with Dengue
Virus Type 4.

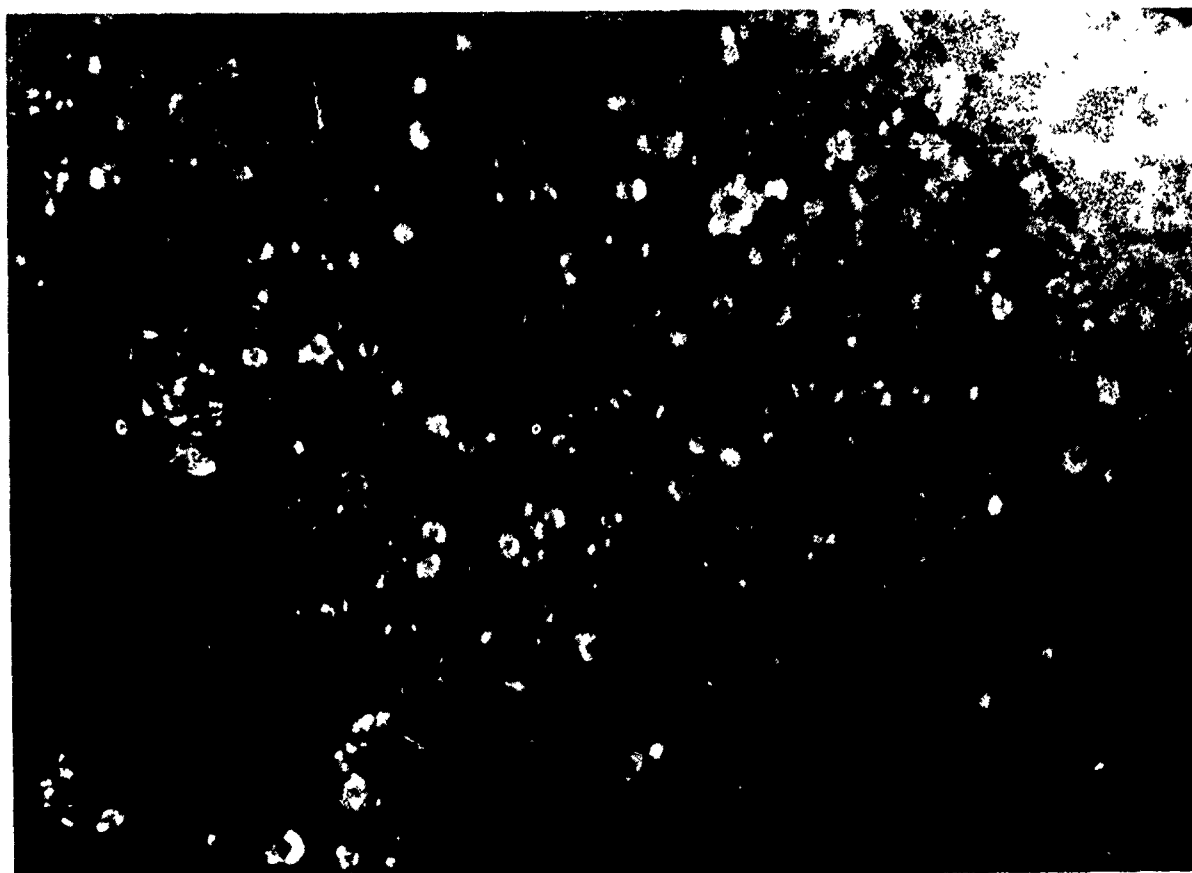
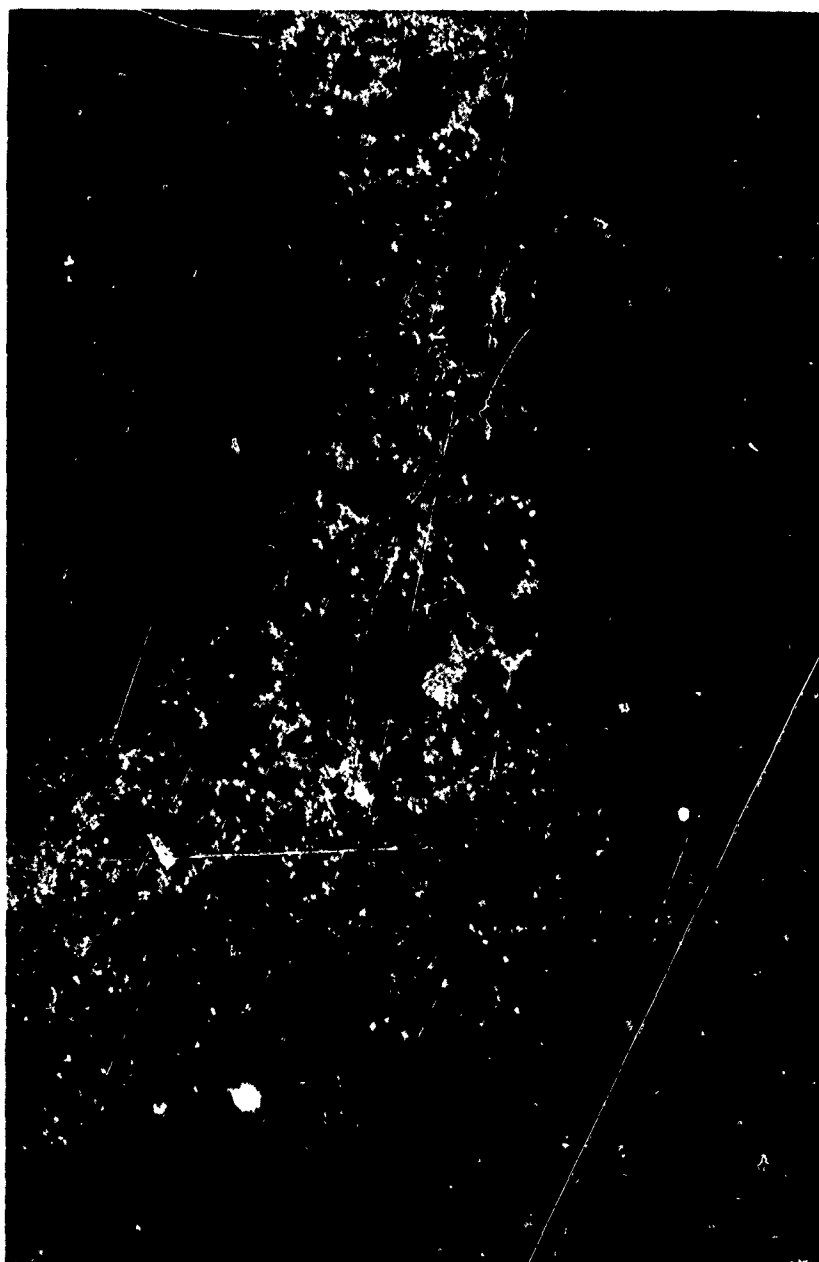


Figure 40 Absence of Perinuclear Fluorescence in Ganglia Cells of Toxorhynchites splendens that was not Inoculated with Dengue Viruses.



The corresponding thorax-abdomen tagmata for the mosquito heads were triturated individually by sonic energy in the presence of 1.5 ml of RPMI 1640 medium, 10% FCS, 500 units of penicillin/ml and 50 ug of streptomycin/ml. Resultant suspensions were centrifuged for 30 minutes at 10,000 rpms in a 4°C centrifuge. Log₁₀ dilutions were prepared of each suspension and assayed for virus in LLC-Mk2 cells by the direct plaque assay technique. Control mosquitoes that received virus diluting medium were assayed as described above. Methods used to assay mosquitoes by the direct fluorescent antibody and by the direct plaque assay for dengue viruses are presented in this Annual Progress Report.

RESULTS: The quantity of dengue virus recovered from individual mosquitoes following intrathoracic inoculation is presented in Tables 73,74,75 and 76. As shown in these tables, dengue virus antigen could not be detected in mosquito heads until or after virus levels had reached maximum titers. An incubation period of 9 days or less was required for the detection of virus antigen in heads of all mosquitoes inoculated with dengue 1 and 2 serotypes. No difference was noted in the time required for dengue virus antigen to appear in mosquito heads for the two strains of dengue 2 virus.

The time required for the appearance of virus antigen in heads of mosquitoes inoculated with dengue 3 and dengue 4 viruses ranged from 9 to 12 days. Of the two dengue 3 virus strains, antigen was detected in 3 of 4 mosquito heads for virus #2797 on day 9 post inoculation, while antigen could not be detected after the same incubation period in heads of mosquitoes that were inoculated with virus #2877. In contrast to dengue virus serotypes 1 and 2, the data indicate that dengue 3 and dengue 4 virus infected T. splendens should be retained for 12 or more days in order to insure the presence of virus antigen in the mosquito heads. Figures 36-40 show the perinuclear fluorescence in ganglia cells of T. splendens infected with dengue 1, 2, 3 and 4 viruses and the absence of fluorescence in cells of mosquito head that was not inoculated with dengue viruses.

The results of a study conducted during 1978 to compare T. splendens with LLC-Mk2 cells for isolating dengue viruses from clinically diagnosed dengue hemorrhagic fever patients are presented in another section of this Annual Progress Report. This is a final report, as the development of the mosquito assay for dengue viruses employing T. splendens has been accomplished.

23. Evaluation of the C6/36 Aedes albopictus Cell Line
as a Substrate for Dengue Virus Growth

OBJECTIVE: To evaluate the utility of the C6/36 Aedes albopictus cell line as a substrate for dengue virus growth.

BACKGROUND: Difficulty is often encountered in attempting to raise infectivity titers of newly isolated dengue virus strains by repeated passage in either LLC-Mk2 cells or suckling mouse brain (SMB). Recently, a new mosquito cell line, the C6/36 Aedes albopictus cell line (137), was developed with the express goal of increased sensitivity and yield specifically for dengue viruses. This line was derived by cloning A. albopictus (Singh) cells in media containing anti-Chukungunya antibody in order to circumvent problems with chronic chikungunya virus infection encountered in other mosquito cell lines. A clone of cells (clone 6) which produced the highest yield of dengue viruses compared to other clones was then selected; this clone was then recloned and the most sensitive sub-clone (C6/36) selected to start the line. The C6/36 cell line is reported to show a cytopathic response to dengue virus infection, and may permit virus yields sufficiently high to allow for virus identification by the plaque reduction neutralization (PRNT) method. We elected to test the line using suckling mouse brain seeds of prototype dengue-1,-2,-3,-4 viruses. To investigate if the line was also sensitive to virus with passage histories other than suckling mouse brain, we also tested a D2 mosquito passage seed and a dengue type 2 LLC-Mk2 tissue culture seed.

METHODS: Viruses: The following virus strains were tested for growth in C6/36 cells: Dengue virus type 2 (Den-2) New Guinea B strain at 29th passage in suckling mouse brain; Den-2 local strain (24742-74) isolated from the plasma of a dengue hemorrhagic fever patient (1974), at the 10th passage in LLC-Mk2 cells; Den-2 local strain passed once in Toxorhynchites mosquitoes; Den-1 Hawaiian strain at the 15th passage in suckling mouse brain (SMB); Den-3 H-87 strain at the 25th passage in SMB; Den-4 H-241 strain at 31st passage in SMB.

Cells: C6/36 cells at the sixth passage level were obtained from Dr. Nathirat Sankavipa at the Virus Research Institute in Bangkok. C6/36 cells were grown in RPMI 1640 with 10% inactivated fetal calf serum at 28°C. LLC-Mk2 cells were grown in Medium-199 with 15% calf serum at 35°C.

Table 77 Yields of LLC-Mk2 Cell Cultures and C6/36 Cell Cultures Inoculated with Dengue 2 Virus Seeds (SMB, LLC-Mk2, or Mosquito).

Virus Strain	Inoculum (PFU/0.2ml)	Day	LLC-Mk2 Yield		C6/36 Yield	
			PFU/0.2ml	HA	PFU/0.2ml	HA
D2SMB	10^5	7	$3 \times 10^{4*}$	0	4.8×10^6	128
		11	-	-	4.5×10^6	256
		15	-	-	$1.2 \times 10^{6*}$	0
	10^4	7	$1.2 \times 10^{3*}$	0	1.3×10^6	32
		11	-	-	$2.1 \times 10^{5*}$	128
		15	-	-	6×10^5	0
	10^3	7	$3 \times 10^{3*}$	0	3×10^5	0
		11	-	-	2.1×10^6	128
		15	-	-	$1.3 \times 10^{6*}$	0
	10^2	7	$3 \times 10^{4*}$	0	9×10^5	0
		11	-	-	$1.2 \times 10^{5*}$	16
		15	-	-	7.5×10^5	0
	10	7	$8 \times 10^{3*}$	0	1.2×10^3	0
		11	-	-	1.5×10^5	0
		13	-	-	5.7×10^4	16
		15	-	-	$6 \times 10^{3*}$	0
	1	7	$6 \times 10^{3*}$	0	1.8×10^2	0
		15	-	-	$4.8 \times 10^{3*}$	0
	10^{-1}	7	0	0	4.5	0
		15	-	-	$6 \times 10^{2*}$	0
D2-LLC-Mk2	5×10^3	7	$4 \times 10^{4*}$	0	3×10^5	128
		11	-	-	3×10^5	128
		15	-	-	$9 \times 10^{6*}$	0
	5×10^2	7	$5.5 \times 10^{4*}$	0	3×10^4	0
		11	-	-	7.5×10^5	32
		15	-	-	$5.1 \times 10^{6*}$	0
	5×10	7	$8 \times 10^{4*}$	0	4.5×10^5	16
		11	-	-	3×10^5	32
		15	-	-	$4.3 \times 10^{6*}$	0

Table 77 Continued

Virus Strain	Inoculum (PFU/0.2ml)	Day	LLC-Mk2 Yield		C6/36 Yield	
			PFU/0.2 ml	HA	PFU/0.2ml	HA
D2-LLC-Mk2	5	7	6×10^4	0	6×10^2	0
		11	-	-	2.7×10^6	32
		15	-	-	$3.9 \times 10^{5*}$	0
	5×10^{-1}	7	$2 \times 10^{4*}$	0	3×10^2	8
		11	-	-	3×10^5	0
		15	-	-	$9 \times 10^{6*}$	0
	5×10^{-2}	7	$6 \times 10^{2*}$	0	10^2	
		11	-	-	10^2	0
		15	-	-	10^{2*}	0
	5×10^{-3}	7	0^*	0	0	0
		11	-	-	0^{2*}	0
		15	-	-	10^{2*}	0
	D2-Mosq. 4×10^3	7	ND		8.7×10^6	64
		11			4.5×10^5	0
		15			3×10^4	0
	4×10^2	7			3.6×10^6	128
		9			6×10^5	0
		13			4.5×10^4	0
		15			Contam	
	4×10	7			1.5×10^4	0
		9			6×10^4	0
		13			7.5×10^4	0
		15			Contam	
	4	7			1.5×10^4	0
		9			1.2×10^4	0
		11			9×10^2	0
		15			$1.5 \times 10^{5*}$	0
	4×10^{-1}	7			1.5×10^{-3}	0
		11			2.4×10^2	0
		15			$6 \times 10^{2*}$	0
	4×10^{-2}	7			0	0
		11			0	0
		15			0	0

* = After one freeze - thaw cycle of cells

Table 78A Yield of C6/36 Cell Cultures Inoculated with
D1 SMB Seed.

Virus Strain	Inoculum PFU/0.2 ml	Day	C6/36 Yield	
			PFU/0.2ml	HA
D1SM	2×10^4	7	5×10^4	64
		9	1×10^4	128
		12	3×10^3	64
		15	2×10^4	0
	2×10^3	7	5×10^4	64
		9	1×10^4	128
		12	6×10^3	64
		15	$1 \times 10^{4*}$	0
	2×10^2	7	2×10^4	32
		9	3×10^4	128
		12	2×10^4	32
		15	Contam	
	2×10	7	1×10^4	32
		9	1×10^4	64
		12	1×10^4	32
		15	1×10^3	0
	2	7	2×10^3	0
		9	2×10^4	0
		12	1×10^3	0
		15	5×10^3	0
	2×10^{-1}	7	10_2	0
		9	$2 \times 10_2$	0
		12	$1 \times 10_2$	0
		15	1×10^3	0
	2×10^{-2}	7	0	0
		9	$5 \times 10_2$	0
		12	1×10^2	0
		15	8×10	0

Table 78B Yield of C6/36 Cell Cultures Inoculated with D3 SMB Seed.

Virus Strain	Inoculum PFU/0.2 ml	Day	C6/36 Yield	
			PFU/0.2 ml	HA
D3SM	2×10^3	7	1×10^4	0
		9	2.5×10^4	0
		12	5×10^3	0
		15	Contam	0
	2×10^2	7	2×10^4	0
		9	2.5×10^4	0
		12	5×10^3	0
		15	Contam	0
	2×10	7	6×10^3	0
		9	1×10^3	0
		12	1.6×10^2	0
		15	Contam	0
	2	7	3×10^4	0
		9	5×10^4	0
		12	2×10^3	0
		15	Contam	0
	2×10^{-1}	7	2×10^4	0
		9	8×10^4	0
		12	6×10^3	0
		15	Contam	0
	2×10^{-2}	7	1.4×10^4	0
		9	5×10^4	0
		12	1×10^3	0
		15	Contam	0
	2×10^{-3}	7	4×10^2	0
		9	1×10^2	0
		12	6.8×10^2	0
		15	Contam	0

Table 78C Yield of C6/36 Cell Cultures Inoculated with D4 SMB Seed.

Virus Strain	Inoculum PFU/0.2ml	Day	C6/36 Yield	
			PFU/0.2 ml	HA
D4 SM	4×10^6	7	3×10^4	8
		9	3×10^4	8
		11	3×10^2	0
	4×10^5	7	5×10^4	8
		9	3×10^5	64
		11	1.8×10^4	0
	4×10^4	7	4×10^4	0
		9	4×10^5	64
		11	6×10^4	0
	4×10^3	7	1.3×10^4	0
		9	5×10^4	0
		11	6×10^4	0
	4×10^2	7	6×10^3	0
		9	3×10^4	0
		11	1.8×10^4	0
	4×10	7	6×10^2	0
		9	1.2×10^4	0
		11	8×10^3	0
	4	7	5×10^2	0
		9	1.6×10^3	0
		11	6×10	0
	4×10^{-1}	7	4.5×10^2	0
		9	2×10^2	0
		11	1×10^2	0
	4×10^{-2}	7	0	0
		9	0	0
		11	0	0

Virus growth curves: Confluent cell monolayers in one ounce bottles were inoculated with 0.1 ml aliquots of selected dilutions of each virus, and then incubated for two hours. The cultures were then replenished with 3 ml RPMI 1640 with 5% inactivated fetal calf serum and placed at 35°C for LLC-Mk2 and 28°C for C6/36 cell cultures. Half of the medium was removed on each sampling day and replaced with new medium. The withdrawn fluid was titrated for infectivity and HA activity.

LLC-Mk2 cultures and C6/36 cultures were subjected to a freeze and thaw cycle in 3 ml of media on day 7 and 15, respectively, and the resultant suspension were then assayed for intracellular virus.

Assays for infectivity titer were done on LLC-Mk2 cells in micro-cultures in 24 well plastic micro-culture plates; HA activity was assayed, without extraction, by standard methods.

RESULTS: Simultaneous titration of the prototype D2 SM seed (D2NGC SM29) on LLC-Mk2 cells and C6/36 cells showed that both the sensitivity and yield of the C6/36 cells were superior for this virus (Table 77). A maximum yield of 10^8 PFU/ml was obtained on C6/36 cells, as compared to a yield of $10^{5.2}$ PFU/ml on LLC-Mk2 cells. In addition, an HA titer of up to 1:256 per 0.025 ml was present in the tissue culture supernatant fluid from the C6/36 cells. Similarly good yields, sensitivity, and HA production were also obtained with the LLC-Mk2 passed virus D2 strain and the mosquito passed D2 strain (Table 77). At similar inoculum doses of approximately 10^3 PFU/0.2 ml, all three strains gave peak yields of 10^5 - 10^7 PFU/ml and peak HAI titers of 1:64 to 1:128.

The C6/36 cell line also supported the growth of D1, D3, and D4 suckling mouse brain seeds; however, peak infectivity titers were lower than we found with D2 and also lower than previously reported by other workers for K1, D3, and D4 (137). Tables 78 A, B, C). Peak HA titers found were 1:128 and 1:64 for K1 and D4 respectively; no HA activity was detected in the cell culture fluid from C6/36 cells inoculated with the D3 SMB seed.

24. Isolation of Dengue Viruses from Aedes aegypti Collected in Bangkok, Thailand

OBJECTIVE: To isolate dengue viruses from Ae. aegypti for studies of the biological and antigenic properties of these viruses.

BACKGROUND: Studies in progress to characterize antigenic and biological properties of wild dengue viruses required field collections of strains of these viruses. According to previous findings, dengue viruses can be isolated from Aedes aegypti collected in and around homes of dengue hemorrhagic fever (DHF) patients (138). The isolation rate observed during investigations conducted at Koh Samui, Thailand, during 1968 was 1 isolate for every 19 female Ae. aegypti assayed. Similar attempts were made during 1976 to isolate dengue viruses from homes of DHF patients in Bangkok; however, the isolation rate was only one dengue virus isolation from over 200 Ae. aegypti collected from homes (139).

MATERIALS AND METHODS: Adults and immature Aedes aegypti and Culex species mosquitoes were collected during the latter months of 1978 and during 1979 in the homes of DHF patients in Bangkok. Male and immature Ae. aegypti were collected for dengue virus transovarial transmission studies described in another section of this Annual Progress Report. The mosquito collecting team included the principal investigator, one entomology technician and a nurse. The collecting period was approximately one hour for each house and all mosquito collections were made between 1330 and 1530 hours. Individual female Ae. aegypti mosquitoes were captured in 2 x 3 cm vials while attempting to feed on members of the collecting team. Culex species were captured while resting on the walls primarily of bathrooms in vials similar to that used for Ae. aegypti.

Mosquitoes were transported to the Laboratory where they were identified and subsequently processed for virus isolation studies. Engorged mosquitoes were retained in individual vials until eggs were laid. A 5% sucrose solution was provided to mosquitoes continuously. Individual mosquitoes were triturated in a sterile tissue grinder in the presence of 1.0 ml of RPMI 1640 medium that contained 10% fetal calf serum (FCS), 500 units of penicillin/ml and 500 ug of streptomycin/ml. The pH of the medium was adjusted to 8.2-8.4 by adding 7.5% NaHCO_3 . Mosquito suspensions were centrifuged for 30 minutes at 4°C at 1000 rpm. The supernatant of each suspension (0.3 ml) was inoculated onto confluent monolayers of LLC-Mk2 cells, two cultures per specimen, one for direct and one for delayed plaque assay. Cell cultures were maintained at 35°C. Some suspensions were also assayed for virus by intrathoracic inoculation of Toxorhynchites splendens that were maintained at 32°C.

Table 79 Summary of Data Concerning the Isolation of Dengue
Viruses from Aedes aegypti, Bangkok, Thailand, 1978-79.

Collection Date	Number of Collections	Number of Mosquitoes	No. Positive/ No. tested
Sept 78	2*	16	2/16
Oct 78	2	36	0/36
Jan 79	4	22	0/22
Feb 79	1	03	2/03
Mar 79	2	07	2/07
Apr 79	3	04	0/04
May 79	2	13	0/13
Jun 79	3	42	0/42
Jul 79	3	25	1/25
	22	168	7/168(1:22.4)**

* Number of houses surveyed for mosquitoes

** One Ae. aegypti per 22.4 mosquitoes tested yielded a dengue virus isolation.

Table 80 Dengue Viruses Isolated from Aedes aegypti Collected in Homes of Dengue Hemorrhagic Fever Patients in Bangkok, Thailand, 1978-1979.

<u>Date Mosquitoes Collected</u>	<u>Houses Number</u>	<u>Cases Number</u>	<u>No. Mosq. Collected</u>	<u>No. Positive/ No. tested</u>	<u>Virus Identity</u>
20 Sept 78	189	D78-091	14	2/14	Den-2
28 Feb 79	008	D79-016	02	2/2	Den-2
16 Mar 79	434/8	D79-024	08	2/8	Den-2
19 Jul 79	35/42	D79-076	18	1/18	Den-2

Table 81 Infectivity Titers of Reference Stocks of Dengue Virus Type-2 Strains in LLC-Mk2 Cells and in Toxorhynchites splendens.

Virus Stock	Date Isolated	Source	Passage History	Infectivity titers LLC-Mk2 cells***
Den-2 (78-091A)	20 Sept78	<u>Ae. aegypti</u>	Ae-2* Ae-1, TS-1**	ND 2.5×10^5
Den-2 (78-091B)	20 Sept78	<u>Ae. aegypti</u>	Ae-2 Ae-1, TS-1	ND 1.0×10^5
Den-2 (79-016-1F)	28 Feb 79	<u>Ae. aegypti</u>	Ae-2 Ae-1, TS-1	3.0×10^4 20×10^4
Den-2 (79-016-2F)	28 Feb 79	<u>Ae. aegypti</u>	Ae-2 Ae-1, TS-1	7.0×10^4 2.0×10^4
Den-2 (79-024-11F)	16 Mar 79	<u>Ae. aegypti</u>	Ae-2 Ae-1, TS-1	1.0×10^4 3.0×10^4
Den-2 (79-024-13F)	16 Mar 79	<u>Ae. aegypti</u>	Ae-2 Ae-1, TS-1	5.0×10^2 3.0×10^3
Den-2 (79-076-1F)	19 Jul 79	<u>Ae. aegypti</u>	Ae-2 Ae-1, TS-1	ND

* Ae-2 indicates that the virus had received one passage in Ae. aegypti after original isolation from Ae. aegypti.

** Ae-1, TS-1 indicate that the virus had received one passage in T. splendens after original isolation from Ae. aegypti.

*** Plaque forming units/0.3 ml.

Virus isolates were identified by plaque reduction neutralization tests employing dengue virus specific antiserum. Stock viruses of each isolate, using original mosquito suspensions, were prepared in Ae. aegypti and in Tx. splendens and if possible, in LLC-Mk2 cells.

RESULTS: Ae. aegypti were collected from a total of 24 houses during September 1978 through July 1979 (Table 79). Of the 24 houses, the patients from 22 were proven dengue virus infection. Collections were made on the average of 6.5 days (range 1-21 days) after patients were admitted to the Children's Hospital. Dengue viruses were isolated from Ae. aegypti collection in 4 of the 22 houses or 1 virus isolated per 5.5 houses. The results of virus isolation attempts from Ae. aegypti are presented in Table 79. A total of seven dengue virus type-2 isolates were made from 168 individually assayed mosquitoes. Multiple isolations were obtained from 3 of the 4 houses that yielded infected mosquitoes. Also, dengue-2 virus was isolated from patients who resided in 2 of the 4 houses. A summary of information concerning virus isolations is presented in Table 80. All mosquitoes were assayed by direct and delayed plaque assay in LLC-Mk2 cells, while 71 of the 157 were assayed in Tx. splendens. The same dengue viruses that were isolated in LLC-Mk2 cells were also isolated in Tx. splendens. Each of the seven isolates were reisolated from the original mosquito suspensions.

Stock viruses of isolates were prepared in Ae. aegypti, Tx. splendens and when possible in LLC-Mk2 cells employing the original mosquitoes as inoculum. A list of stock viruses and history of each stock is presented in Table 81.

25. Longevity and Developmental Studies of Aedes aegypti

OBJECTIVE: To determine the seasonal longevity and developmental rates of Aedes aegypti.

BACKGROUND: Previous population dynamics studies of Aedes aegypti in Bangkok did not reveal a significant relationship between the survival rate of this mosquito and the seasonal variation in prevalence of human dengue virus infections (140). Recent observations suggest that the prevalence of dengue virus infections during the rainy season may be related to environmental factors occurring during the preceding cool season (141). Since the development time and survival of Ae. aegypti are markedly influenced by temperature (142, 143), field and laboratory investigations on temperature and humidity were initiated to reconsider this aspect of the population dynamics of aegypti in Bangkok.

METHODS: Aedes aegypti longevity and developmental studies were conducted in a house located in the Din Daeng study area and in environmental chambers in the Department of Medical Entomology, AFRIMS. Hereafter the house will be referred to as the field house. Aedes aegypti larvae were collected from 14 houses in the Din Daeng study area (23) during the latter part of December 1978, and again during the latter part of May 1979, to provide mosquitoes for studies during the cool and hot seasons, respectively. Larvae were reared in the field house and adults were allowed to feed on hamsters to provide F₁ eggs. Eggs were hatched and 200 larvae were transferred to each of 3 plastic trays (20 cm x 26 cm x 4.5 cm) that contained 1000 ml of water. In addition, 200 larvae were placed in each of 4 ceramic water (ong) jars (28 l.) that contained 12 l. of water. Before adding water to the 4 ong jars, each jar was washed thoroughly and scoured with a steel brush to eliminate any extraneous aegypti eggs. Water came from a large ong jar used by a family for storing tap water for everyday use.

Aedes aegypti found in the water source jar were removed from the water before it was added to the experimental water containers. A thin layer of sediment settled out of the water in each experimental container. Sediment was also observed in ong jars in houses throughout in Din Daeng study area and in larval pans in the 7th floor insectary of the main AFRIMS laboratory. Apparently, the sediment comes from the tap water, and it probably serves as a food source for aegypti larvae. Beside testing the survival of 200 larvae each in 2 test ong jars with this water (without added food), one gram of ground mouse chow was placed in each of the 3 trays and the 2 remaining ong jars containing larvae.

Each day the water temperature was recorded and the containers were checked for pupae. Pupae were removed daily, counted and transferred to water in screened paper cups for retaining emerging adults.

The longevity studies were initiated with newly emerged F₁ generation aegypti. Males and engorged and unengorged females were transferred to paper cups, 10 per cup and placed on the floor along the walls of each room of the field house. Cotton saturated with a 5% sucrose solution was provided continuously to mosquitoes. An oviposition substrate was provided for some of the engorged mosquitoes. The number of surviving mosquitoes was recorded daily. A hygrothermograph was used for recording temperature and humidity.

Aedes aegypti eggs from the same mosquitoes that provided eggs for the studies in the field house were used as a source of mosquitoes for laboratory studies. In addition, aegypti from three different laboratory colonies were employed. The latter mosquitoes were included for comparative data on the rate of development and to study the effects of temperature on the isoenzyme profile of the different strains as reported elsewhere in this Annual Report. Eggs were hatched and 200 larvae each were placed in plastic trays, each containing 1000 ml of water. One gram of ground mouse chow was added to each tray.

Larvae were placed in environmental chambers that were maintained at approximately 20° and 35°C. Selection of temperature was based on extreme minimum and maximum temperatures that occur in Bangkok. Daily records were maintained for temperatures, the number of pupae and the number of adults that emerged for each strain as described for the Din Daeng field house studies.

RESULTS: The development and longevity of immature aegypti reared in the Din Daeng field house during the winter season are summarized in Table 82. The development of first stage larvae to adults in ong jars with food was rapid, with 50% of males and 50% of females emerging within 6.4 to 6.9 days. In the jars with food, the survival rate from first stage larva to adult was high, 0.92 and 0.85 for each of two replicates of 200 larvae. In contrast, the time required for emergence of 50% of adults from larvae reared in one ong jar without food, ranged from 15 to 18.1 days for males and females, respectively. Also, the survival (0.56) of immatures was much lower than that of larvae reared in jars supplemented with food. Developmental studies of 200 Ae. aegypti larvae in the other ong jar not supplemented with food were aborted due to predation by Toxorhynchites splendens larvae.

Figures 41 and 42 shows the pattern of development and of emergence of immatures reared with and without food added to the ong jars. The prolonged period and sporadic pattern of emergence of aegypti reared in the jar not supplemented with food is consistent with data for emerging adults trapped during population density studies reported elsewhere in this Annual Report. This suggests that the availability of food is an important determinant of the rate of development and productivity of aegypti in the ong jars in the Din Daeng study area. The rate of development of immature aegypti reared in trays was approximately one day longer and longevity was slightly lower than that for mosquitoes reared in ong jars supplemented with food. These differences may have resulted from crowding in the different volumes of water in trays as compared to jars.

Table 82 The rate of development of F_1 generation Aedes aegypti in the Din Daeng Field House during the cool season, 23 January-3 March, 1979¹

Mosquitoes ²	No. of 1st stage larvae	Time to Pupation (50%) ³	Time to Emergence(50%) ³		Period Egg to Last Adult (days)	% Survival To Adult
			Males	Females		
DDFH (ong jar-food)	200	4.7	6.4	6.8	21	92
DDFH (ong jar-food)	200	4.7	6.6	6.9	18	85
DDFH (ong jar-no food)	200	14.6	15.0	18.1	44	56
DDFH (Tray-food)	200	5.9	9.0	8.0	12	85
DDFH (Tray-food)	200	5.5	7.5	7.7	Not determined	71
DDFH (Tray-food)	200	6.0	7.7	8.0	14	75
DDFH (Tray-food)	200	5.6	7.6	7.8	Not determined	77

¹ Temperature mean and range for the room was 30°C (24-33°C), and the temperature mean and range for the water was 26.6 (24-28°C)

² Aedes aegypti were reared from eggs of females that originated from larvae collected in the Din Daeng study area during December and January 1978 and 1979.

³ Days past hatching of eggs that 50% of mosquitoes had pupated or emerged.

Table 83 Survival of F_1 generation Aedes aegypti in the Din Daeng field house during the cool and hot seasons¹.

Sex/condition ²	Season	Number of Mosquitoes	Survival (Days)	
			Mean ³	Range
♀♀ (engorged)	cool	90	17.5	2-25
♀♀ (engorged)	hot	150	8.0	2-21
♀♀ (engorged) ⁴	cool	30	27.0	7-39
♀♀ (engorged) ⁴	hot	30	23.0	1-30
♀♀ (unengorged)	cool	60	17.5	2-25
♀♀ (unengorged)	hot	60	12.5	2-26
♂♂	cool	40	7.8	1-30
♂♂	hot	40	6.5	1-23

¹ Cool Season: Mean maximum and minimum temperatures were 32.2° and 27.8°C, respectively, range-maximum 30.6° -33.3°C, minimum 23.8° -32.2°C; and the mean maximum and minimum humidities were 97% and 68% respectively, range-maximum 80%-100%, minimum 46%-80%.

Hot Season: Mean maximum and minimum were 33.3° and 28.9°C, respectively, range-maximum 30.6° -35°C, minimum 26.6° -30.6°C; and the mean maximum and minimum humidities were 92% and 67%, respectively, range-maximum 76%-100%, minimum 56%-89%.

² Sugar water provided to all mosquitoes.

³ Age to which 50% of mosquitoes survived.

⁴ Oviposition substrate provided.

Table 84 The rate of development of different strains of Aedes aegypti at different temperatures in the laboratory, AFRIMS, 11 January-23 January 1979.

Strains	Temperature	No. of Larvae	Time to pupation(days) (50%)	Time to emergence(days) (50%)		Period (days) Egg to last adult	%survival to adult
				Males	Females		
Colony #1 ¹	19.8 (19-20)	200	12.1	16.0	15.2	28	51
Colony #1	34.7 (30-38)	200	3.5	5.0	5.5	18	80
Colony #3 ²	19.8 (19-20)	200	12.0	15.0	17.3	29	65
Colony #3	34.7 (30-38)	200	5.0	6.0	7.3	16	92
Colony #4 ³	19.8 (19-20)	200	11.3	14.8	17.5	21	58
Colony #4	34.7 (30-38)	200	3.7	4.9	5.9	15	78
DDFH ⁴	19.8 (19-20)	200	13.1	16.7	18.0	36	46
DDFH	34.7 (30-38)	200	5.0	6.5	6.6	12	88

- 1 Colony established during 1968 from unknown specimens collected at Koh Samui.
- 2 Colony established during 1977 from larvae collected in Bangkok, Thailand, F-19 generation
- 3 Colony established during 1977 from 2 adults(♂+♀) collected in Bangkok, F-10 generation
- 4 Colony established during 1979 from larvae collected in Din Daeng, Bangkok, Thailand, F-1 generation.

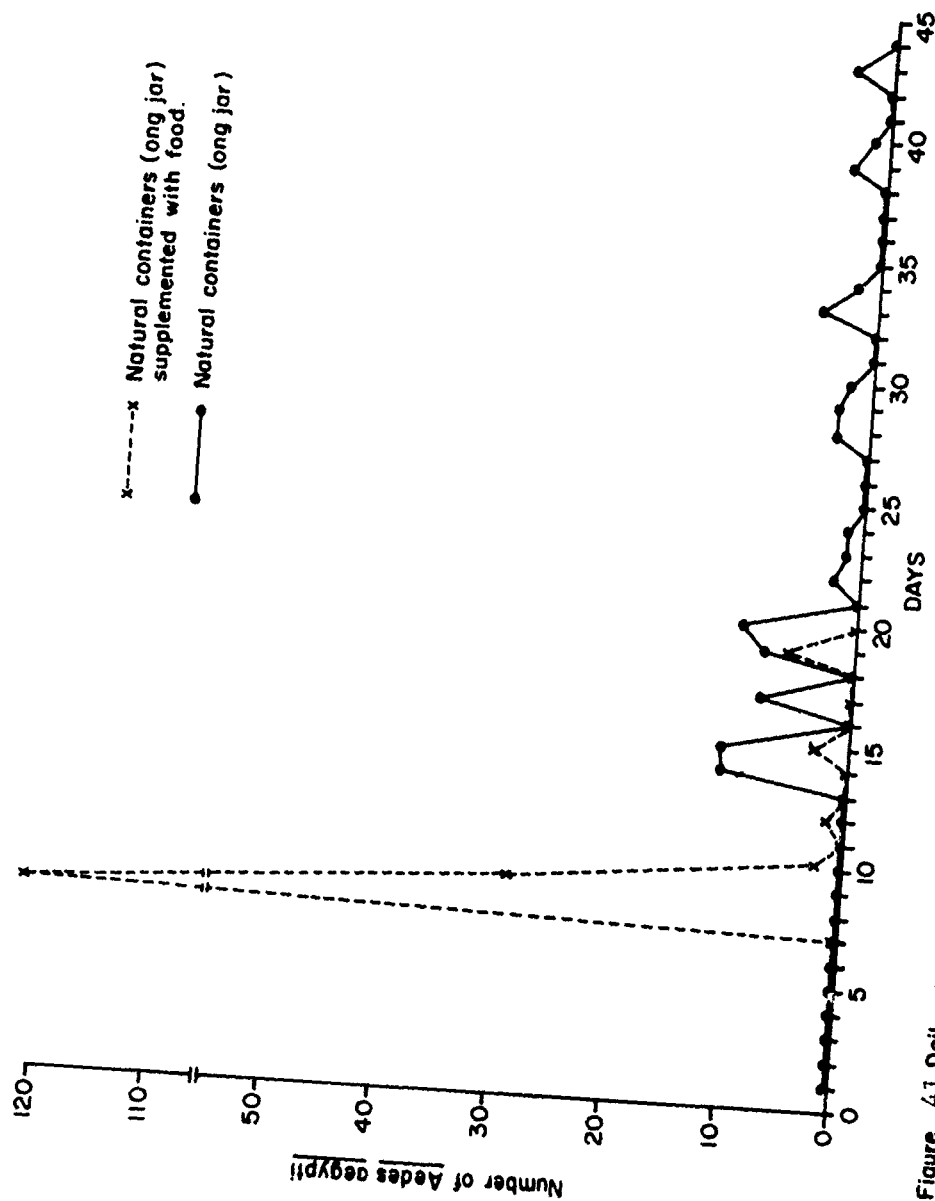


Figure 41 Daily emergence of aedes aegypti that developed from larvae reared in a natural container and in a natural container supplemented with food.

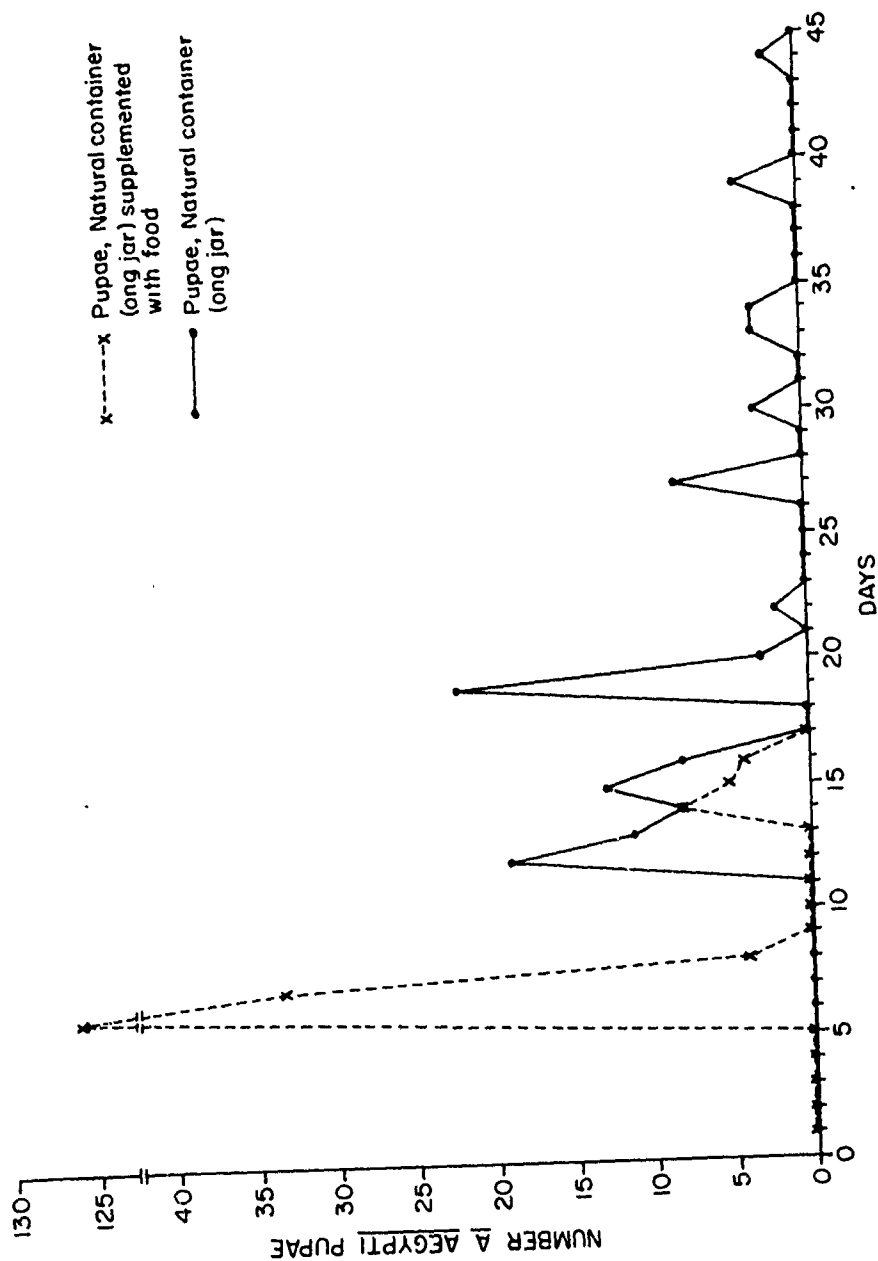


Figure 4.2 Daily pupation of *Aedes aegypti* larvae that were reared in a natural container and in a natural container supplemented with food

The results of longevity studies for adult aegypti during the cool and hot seasons are presented in Table 83. Mean longevity was longer for both male and female mosquitoes during the cool season. The greatest difference was observed for engorged and unengorged female aegypti that were not provided an oviposition substrate. Engorged mosquitoes of both the cool and hot season exhibited strikingly higher longevity than the other females. In addition, the range of longevity for the former mosquitoes was longer than the other females. Mean longevity of males was approximately the same for both seasons. Although mean longevity for males was lower than that of most groups of females, the range of longevity of males was comparable to that of females.

A marked difference was noted in the rate of development and longevity of aegypti reared at different temperatures in the laboratory (Table 84. The time required for the emergence of 50% of adults of different strains of mosquitoes was approximately three times longer for mosquitoes reared at 20°C. Only slight variation was observed in the development rates among the different strains reared at 20°C, with the Din Daeng strains requiring the longest period of time. Survival rates from first stage larvae to adults were also lower for mosquitoes reared at the lower temperature. The development and survival rates of mosquitoes reared at the higher temperature were somewhat similar to those observed for mosquitoes reared in the Din Daeng field house at 30°C, except for the groups reared in the ong jar that was not supplemental with food. The results for the latter were similar to those for mosquitoes reared at the lower temperature in the laboratory. Additional studies will be conducted to further define the seasonal development and survival of Ae. aegypti.

26. Effects of Temperature on the In Vitro Replication of Dengue Viruses

OBJECTIVE:

1. To determine the kinetics of replication and the patterns of thermo-inactivation, at different temperatures, of selected dengue viruses that originated from dengue virus infected humans and mosquitoes.
2. To determine the plating efficiency, at different temperatures, of dengue viruses isolated directly from humans with a clinical diagnosis of dengue hemorrhagic fever.

BACKGROUND: This is a continuation of an investigation that was initiated during 1978 to determine if the virulence properties of dengue viruses could be assessed on the basis of the degree of replication at different temperatures. Preliminary findings indicated that the pattern of replication of selected serotypes and strains of dengue viruses in LLC-Mk2 cells at different temperatures did not differ significantly. Studies to define the plating efficiency of wild dengue viruses at different temperatures were hindered by the failure to reisolate viruses from the original specimens. However, limited data suggested that the plating efficiency of dengue virus type-3 strains was greater at 32°C than at 35 and 39°C. This report includes the results of observations made on additional serotypes and strains of dengue viruses. In addition, selected serotypes and strains of dengue viruses were characterized in regard to their rates of thermo-inactivation at different temperatures.

METHODS: Dengue viruses employed in virus replication and thermo-inactivation studies originated from humans or from Aedes aegypti mosquitoes. Stock viruses were prepared in LLC-Mk2 cells, suckling mice, and in Toxorhynchites splendens. Viruses of human origin included serotypes and strains that were associated with human disease that varied in severity from grades I to IV. A list of dengue viruses selected for investigation is presented in Table 85.

The plating efficiency of dengue viruses was determined on the basis of the number of plaque forming units (PFU) obtained directly from plasma and cellular components of the blood of dengue fever and dengue hemorrhagic fever (DHF) patients diagnosed at Children's Hospital during 1978. Suspensions of plasma and cellular components were assayed by the direct plaque technique in LLC-Mk2 cells at 32, 35, and 39°C. Separation of cellular components into leukocyte subpopulations and platelets was performed as described elsewhere in this Annual Report. Viruses were identified by plaque reduction neutralization tests (PRNTS) employing dengue virus specific antisera prepared in Rhesus monkeys.

Virus replication curves were performed for dengue viruses at 32, 35, and 37°C in LLC-Mk2 cells. Screw cap tubes were seeded with 1.0 ml of a cell suspension that contained $10^{5.0}$ cells per ml. After 4 days at 37°C, medium-free monolayers were inoculated with a dilution of virus to yield a multiplicity of infection (MOI) of 0.1. Cultures with inoculum were incubated for one hour at 32, 35, and 37°C. After the absorption period, 1.0 ml of RPMI 1640 medium was added to each culture. Medium contained 10% fetal calf serum (FCS), 200 units of penicillin per ml and 200 ug per ml of streptomycin.

At 12 hours post inoculation and at 12 hour intervals thereafter, 3 cultures per temperature were harvested for virus assay. After removing cells from the tube wall by vigorous agitation with glass head, 0.5 ml of FBS was added to each suspension. Aliquots of 0.5 ml of each suspension were stored at -70°C . Suspensions were assayed for virus by a micro-plaque assay employing 24 hole disposable plastic plates. Log_{10} dilutions were made in RPMI 1640 medium and inoculated onto medium-free cell monolayers, 0.05 ml per well. After a 60 minute absorption at 35°C , each culture received 0.5 ml of an agar overlay. Cells were incubated at 35°C for 5 days and 0.5 ml of a second agar overlay that contained a 1:1000 dilution of neutral red was added to each cell culture. PFUs were counted and recorded on day 6 post inoculation.

Thermo-inactivation rate of the infectivity of dengue viruses was determined at 37 and 41°C . One ml of each stock virus suspension was added to 9.0 ml of preheated RPMI 1640 medium and/or human serum that was shown to be free of dengue virus antibody by plaque reduction neutralization test (PRNT). A 0.5 ml aliquot of each virus suspension was obtained immediately and at 2, 4, and 8 hours of incubation. Log_{10} dilutions were assayed for virus by PRNT in LLC-Mk2 cells.

RESULTS: The infectivity titers attained by strains of dengue virus types 1, 2, 3 and 4 in LLC-Mk2 cells that were maintained at 32, 35, and 37°C are presented in Figures 43, 44, 45 and 46. Strains of dengue virus type-2 and 4 exhibited comparable titers at each temperature. The magnitude of replication of the grade I strain of dengue virus type 1 was slightly higher than that of grade II strain at 32, 35, and 37°C . Of the two strains of dengue virus type-3 the grade IV strain exhibited significantly higher titers than that of the grade I strain. In addition, the latent period of the grade I strains was 48 to 60 hours longer than that of grade IV strains.

As a result of problems encountered in the preparation of stock viruses, both cell culture and mouse brain propagated viruses were employed in virus replication curves. The possibility that the source of the virus stocks influenced the results was considered by conducting virus replication studies with dengue virus type-1 and type-2 stocks that were prepared in both cell culture and suckling mice. As shown in Figures 47 and 48 the latent period and infectivity titers exhibited by dengue virus types-1 and -2 were comparable regardless of the source of the stock virus.

Since the thermo-stability of the infective properties of viruses can be affected by the type and content of media, an experiment

TABLE 85. Dengue virus serotypes and strains employed in virus replication and thermolability studies.

Dengue	Virus	Host-Passage	Grade of Illness	LLC-Mk ₂ Cells (PFU/1.0 ml)	SMLD ₅₀ **
Den-1	75-001	Mk ₂ -5	I	5.7*	5.7
	-001	SM-3	I	6.1	6.3
	74-112	Mk ₂ -5	IV	5.5	5.2
	-112	SM-3	IV	7.2	7.2
Den-2	77-132	Mk ₂ -4	I	4.3	NA
	3379	SM-5	II	6.5	8.3
	77-248	Mk ₂ -4	IV	6.8	5.2
	-248	SM-3	IV	7.9	8.6
Den-3	77-2877	SM-5	I	6.3	5.2
	77-2797	SM-5	IV	6.2	7.4
Den-4	77-092	Mk ₂ -4	I	3.7	3.5
	-092	SM-3	I	7.3	7.1
	77-380	Mk ₂ -4	III	4.2	NA
Den-2	189-17A	<u>T. splendens</u> -1	-	3.7	-
Den-2	189-17A	Mk ₂ -1	-	4.4	-
Den-2	189-18A	<u>T. splendens</u> -1	-	4.0	-
Den-2	189-18A	Mk ₂ -1	-	4.0	-

* = Log₁₀ Plaque forming unit/1.0 ml

** = Suckling mouse lethal dose/1.0 ml
50

NA = Not adapted to suckling mice

T. splendens = Toxorhynchites splendens

SM = Suckling mice

Mk₂ = Monkey kidney cells

Table 86 The yield of dengue virus type 2 obtained from plasma and leukocyte fractions that were assayed at difference temperatures.

Specimen Number of	Plasma		Mononuclear Cells		Polymorphonuclear Cells		Grade of virus Illness		serotype
	32°	35°	37°	32°	35°	37°	32°	35°	
D-78-078	00	04*	00	TNTC	TNTC	TNTC	04	00	Den-2
D-78-081	TNTC	00	00	---	---	---	00	05	Chikungunya
D-89-091	-	-	-	08	82	66	-	-	Den-2
D-78-099	TNTC	TNTC	TNTC	-	-	-	-	-	Den-2
D-78-112	00	52	53	00	01	04	00	00	Den-2
D-78-114	TNTC	TNTC	TNTC	24	TNTC	TNTC	03	05	Den-2
D-78-117	C	TNTC	TNTC	08	TNTC	10	?	TNTC	Den-2
D-78-132	03	05	04	04	01	00	-	-	Den-2
D-78-133	09	37	40	00	01	00	03	00	Den-2
D-78-135	28	10	20	80	10	17	22	10	Den-2
D-78-136	-	-	-	00	TNTC	162	-	-	Den-2
D-78-156	-	-	-	00	03	00	-	-	Den-2
D-78-157	00	74	59	00	02	04	-	-	Den-2
D-78-159	00	28	27	00	03	00	-	-	Den-2
D-78-168	-	-	-	00	04	00	-	-	Den-2
Total	7	10	9	7	13	8	4	4	3

* Plaque forming units/0.3 ml.

** Not done.

TABLE 87 The distribution of dengue virus type-2 strain isolations by source and by temperature of primary isolation culture.

Source of virus	Temperature of primary isolation culture						Total
	32-35-37°C	32-35°C	35-37°C	32°C	35°C	37°C	
Plasma*	5	0	3	0	1	0	09
Mononuclear cells	5	1	3	0	4	0	13
Pc polymorphonuclear cells*	1	1	0	2	1	1	06

* D-78-117 isolate not included as results were inconclusive for 32°C

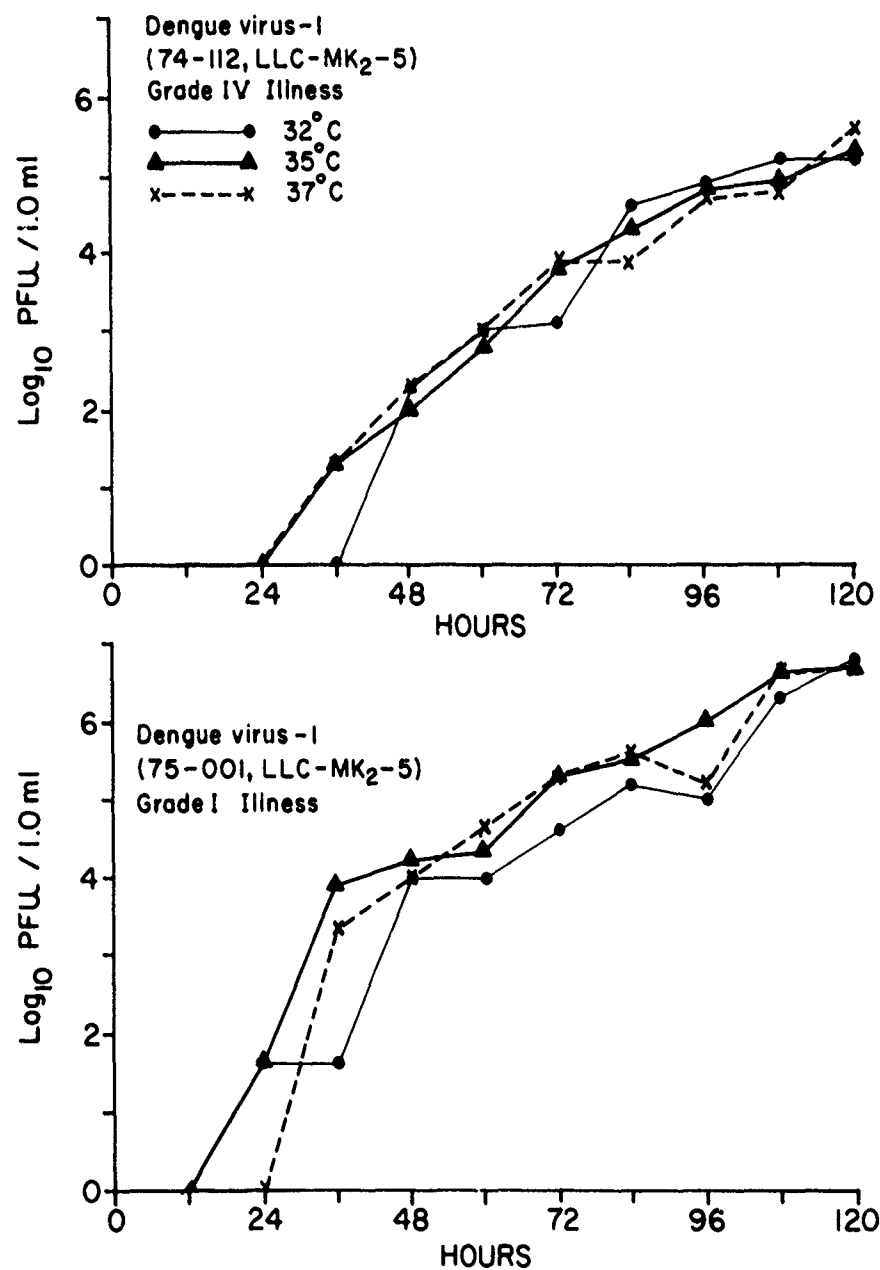


Figure 4? Replication of dengue virus type I strains in LLC-MK₂ cells at different temperatures.

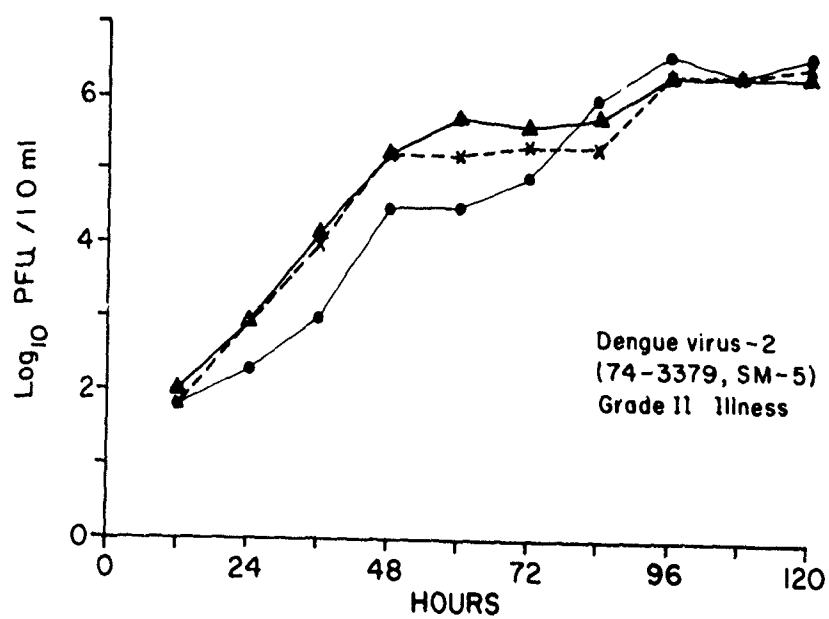
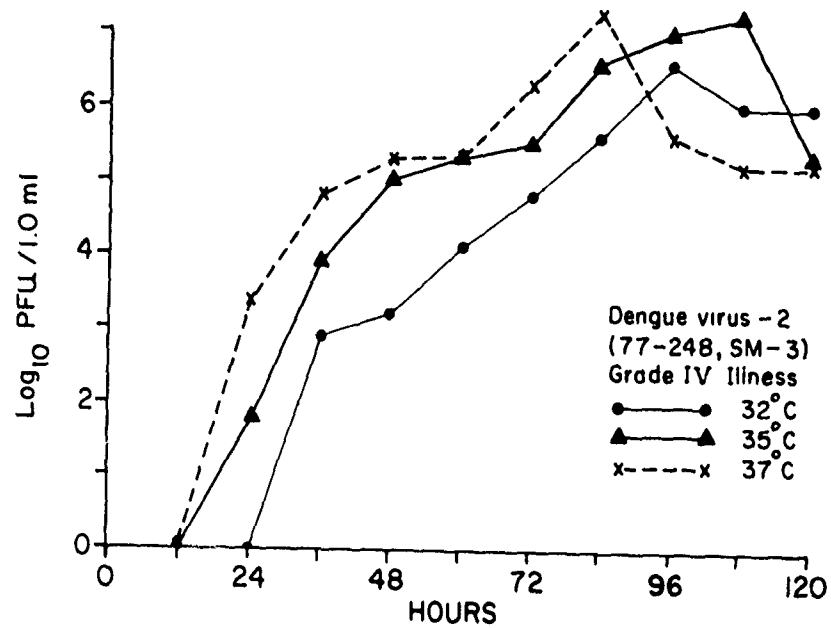


Figure 4/ Replication of dengue virus type 2 strains in LLC-MK₂ cells at different temperatures.

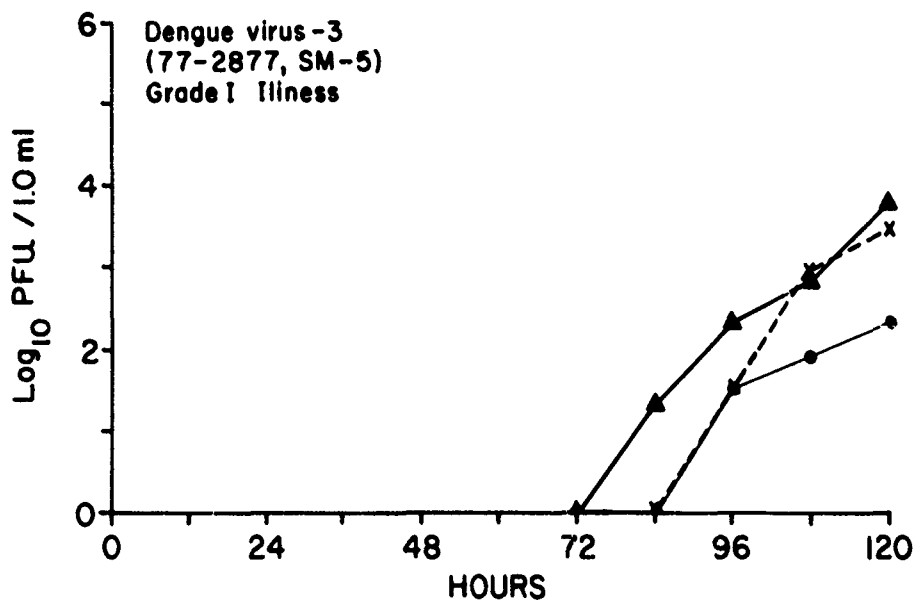
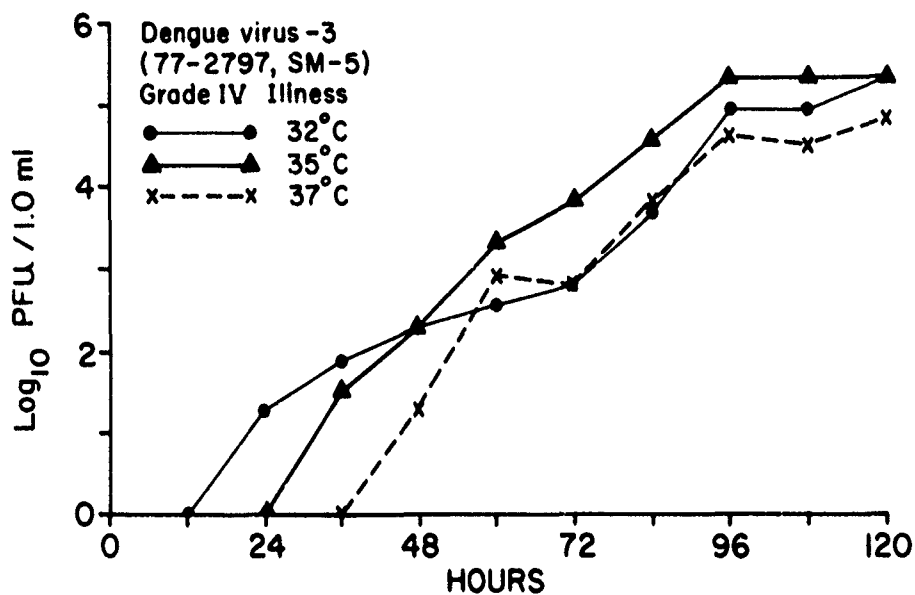


Figure 45 Replication of dengue virus type 3 strains in LLC-MK₂ cells at different temperatures.

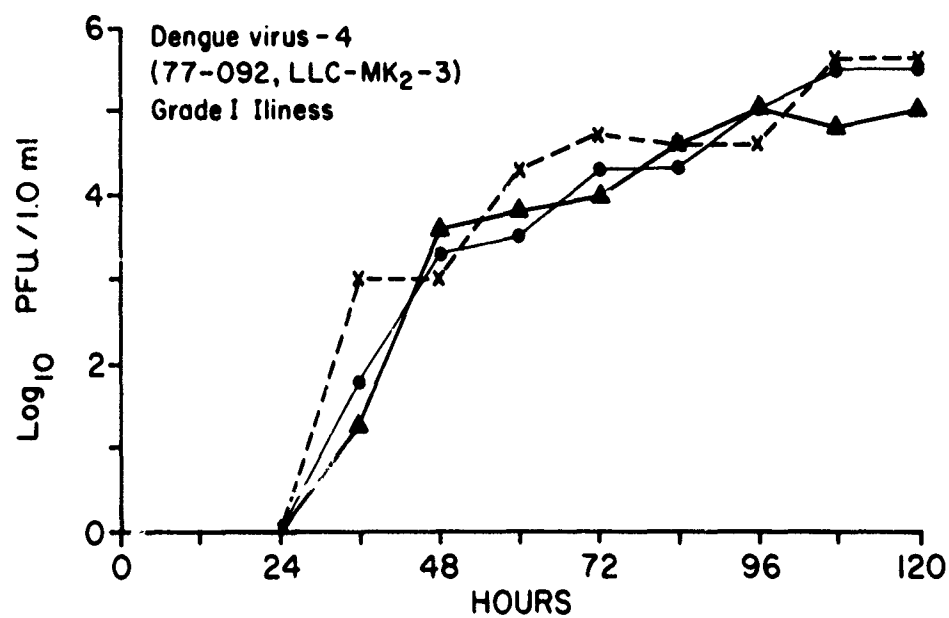
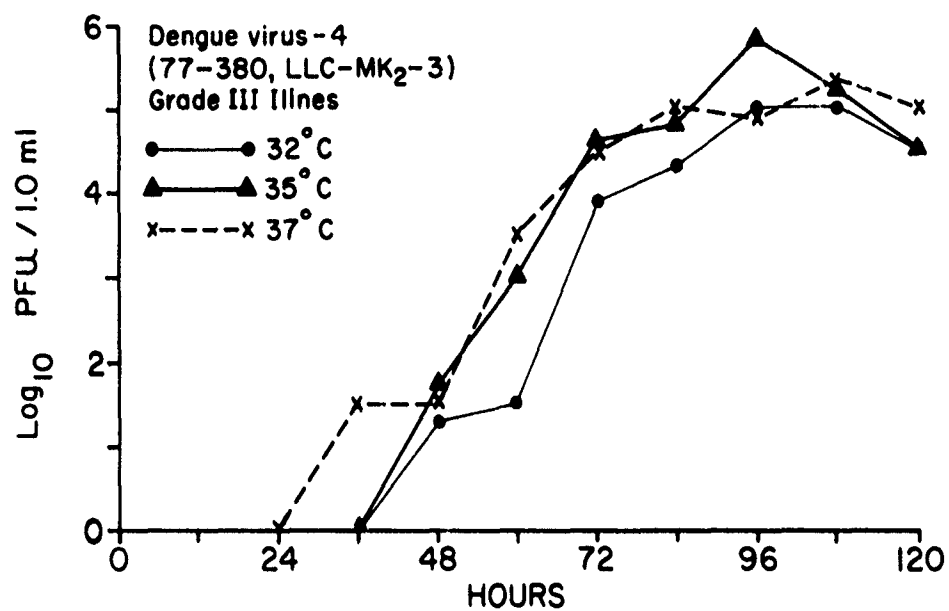


Figure 4. Replication of dengue virus type 4 strains in LLC-MK₂ cells at different temperatures.

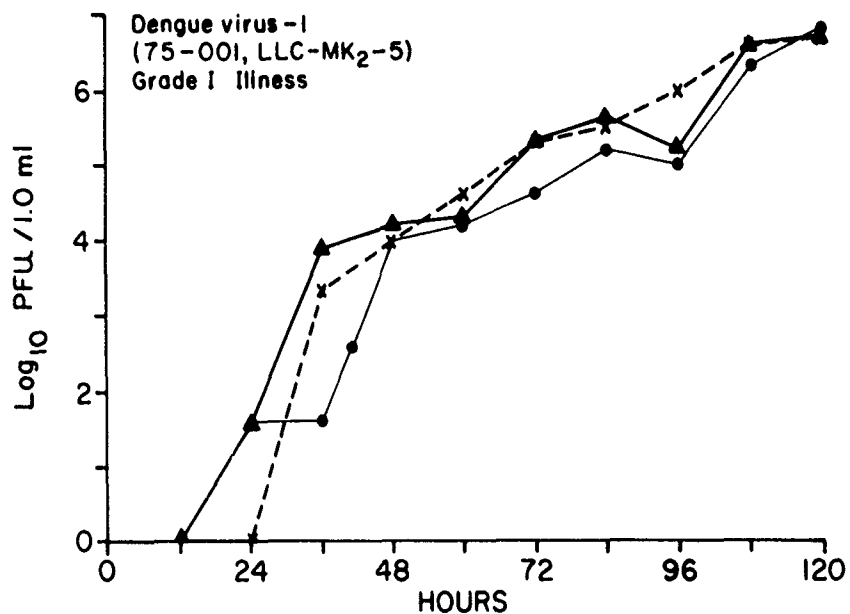
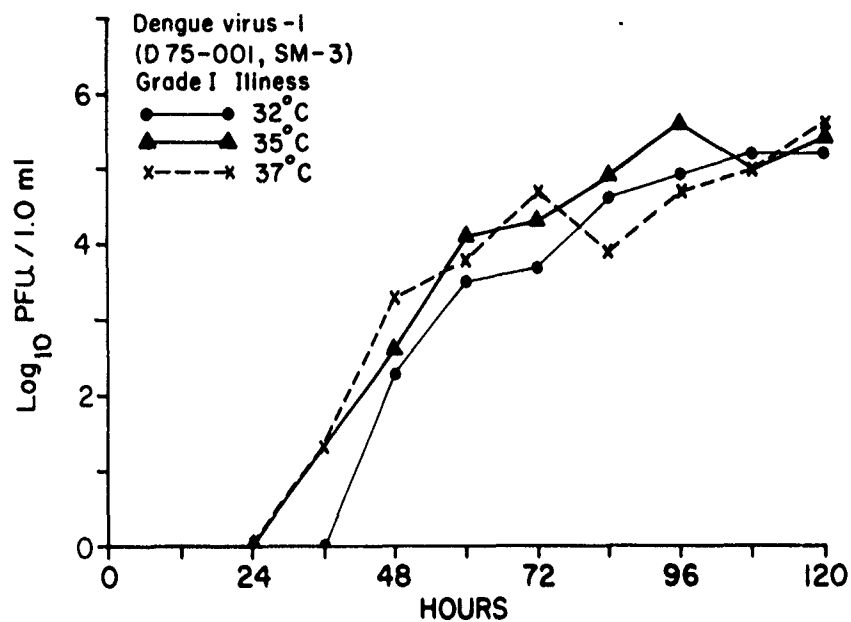


Figure 47 Replication of dengue virus type I suckling mouse brain passaged seed and a cell culture propagated seed virus, in LLC-MK₂ cells at different temperatures.

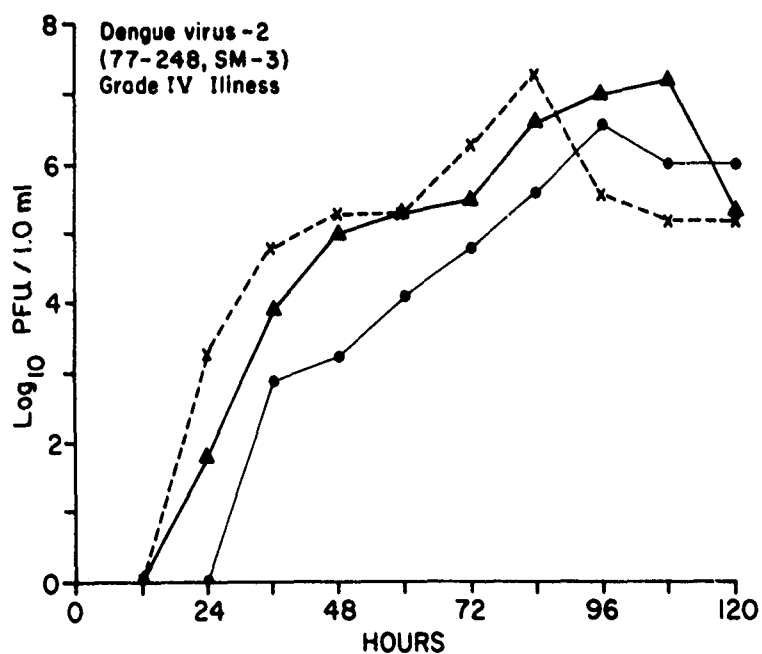
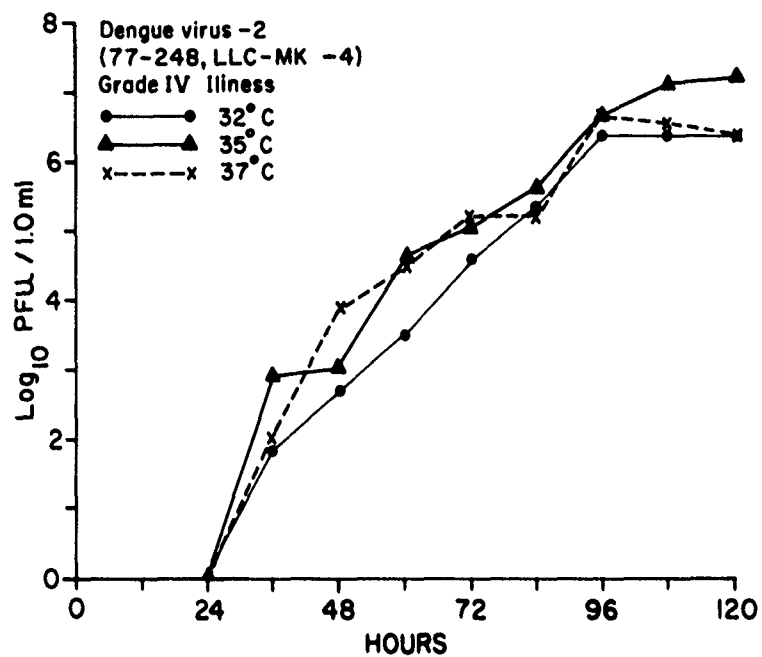


Figure 48 Replication of dengue virus 2 suckling mouse brain passaged seed and a cell culture propagated seed virus, in LLC-MK₂ cells at different temperatures.

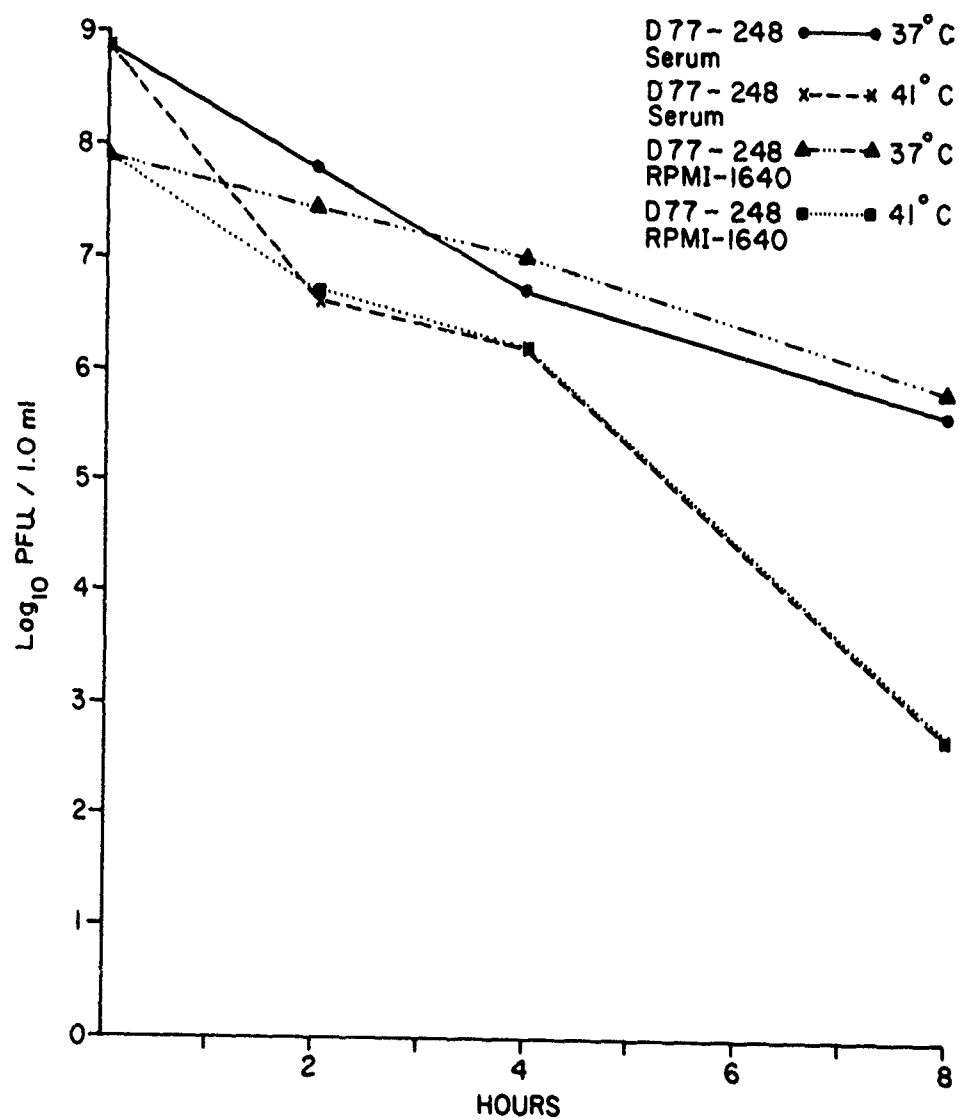


Figure 49 Thermolability of dengue virus type 2 in human serum and in RPMI 1640, 10 % fetal calf serum medium.

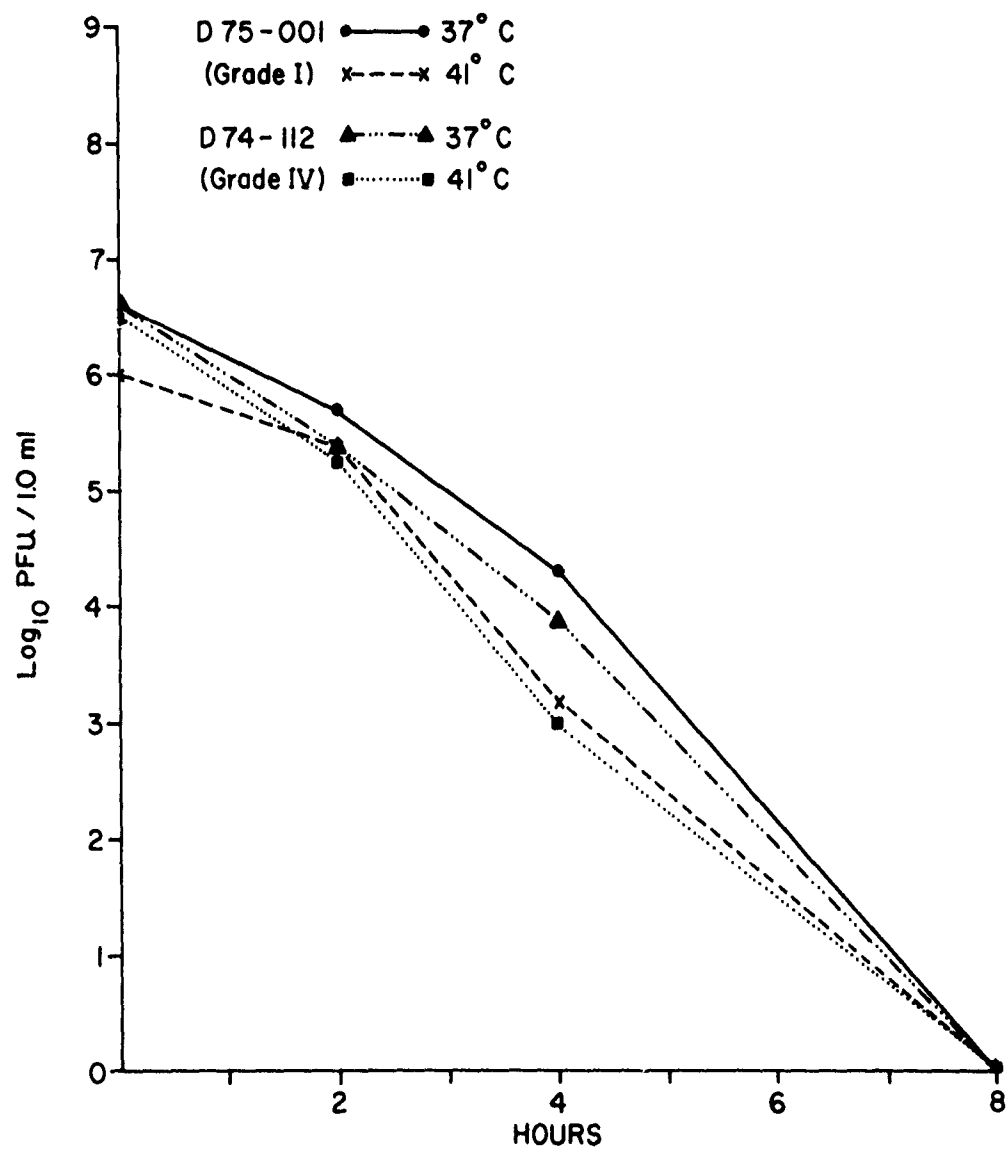


Figure 50 Thermolability of dengue virus type I strains.

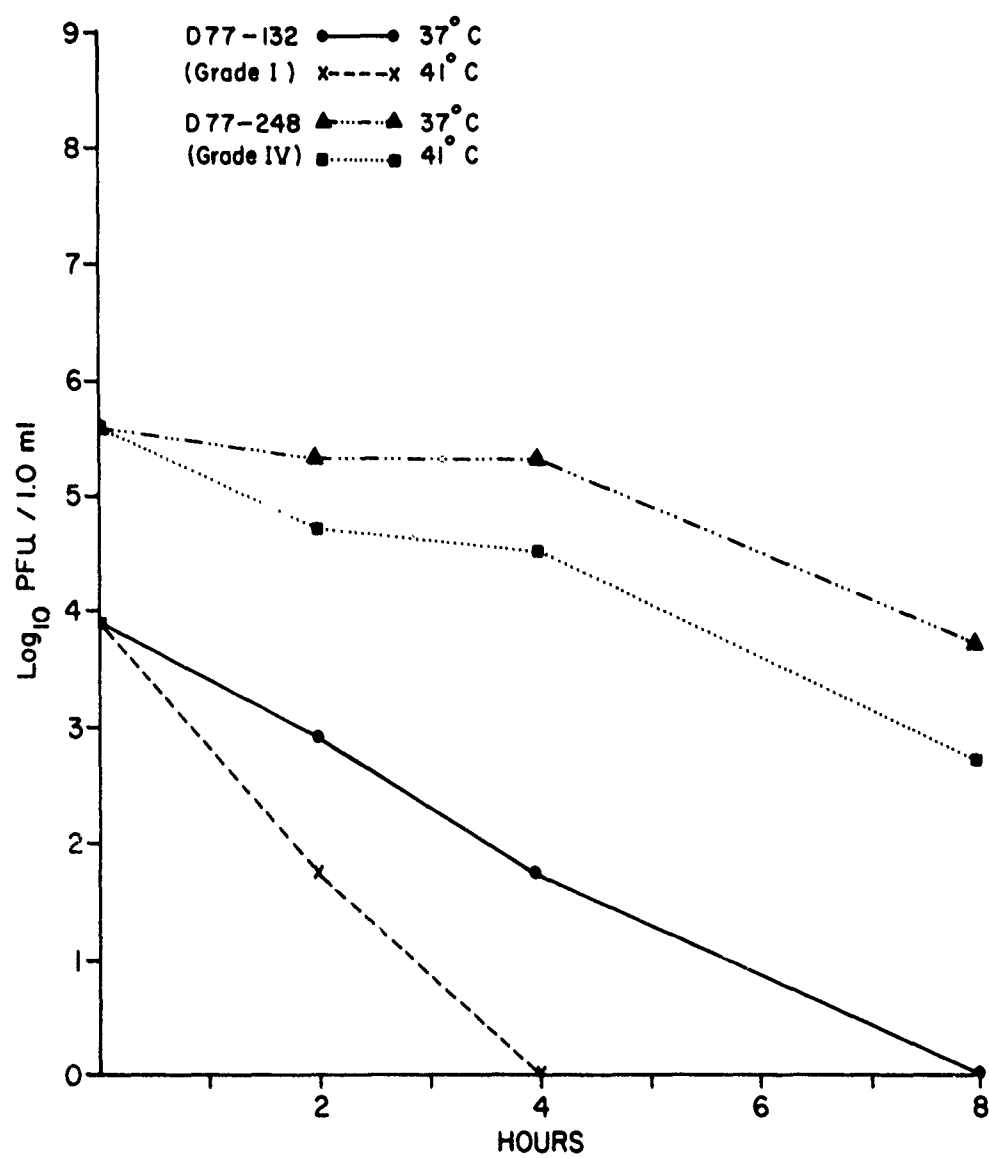


Figure 51 Thermolability of dengue virus type 2 strains.

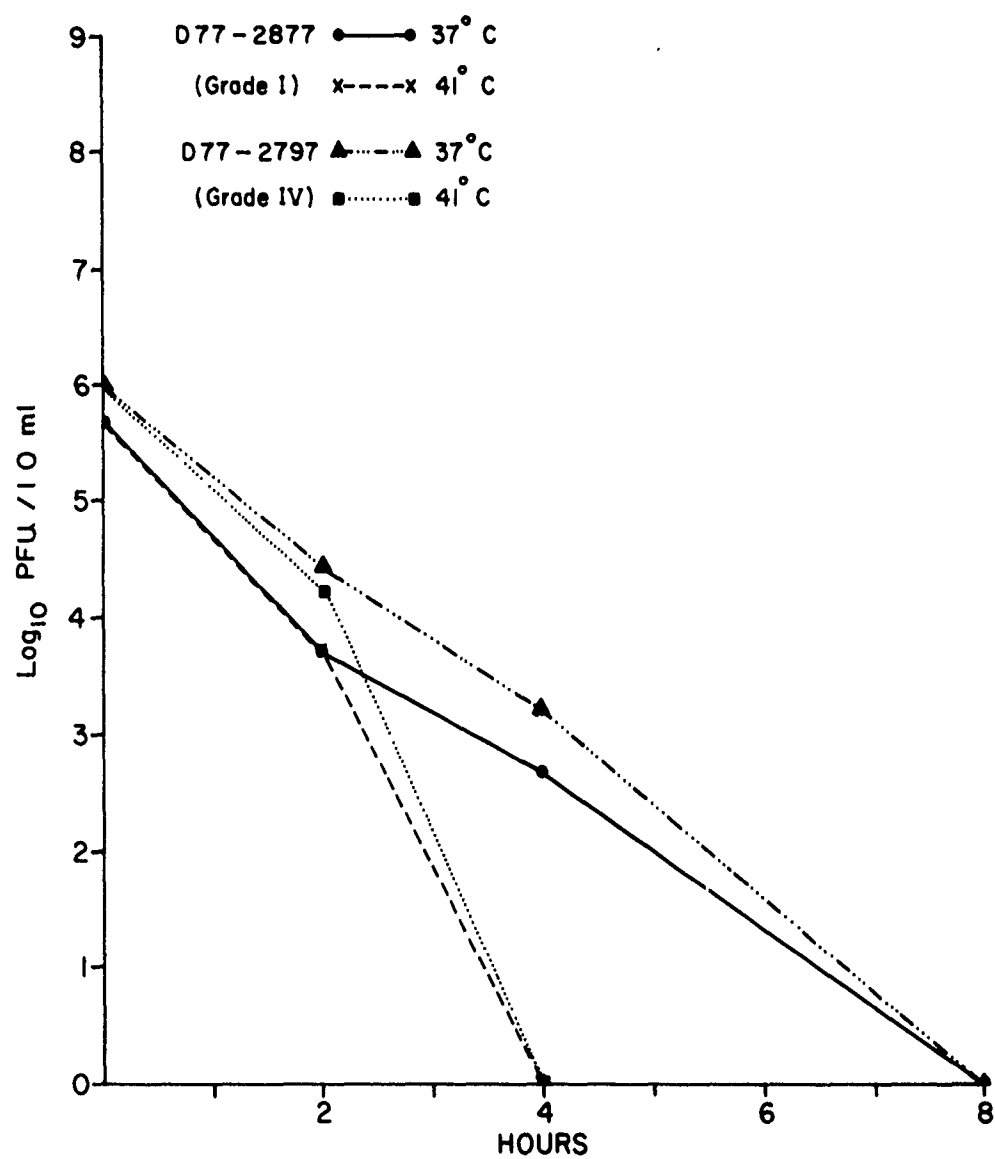


Figure 52 Thermolability of dengue virus type 3 strains.

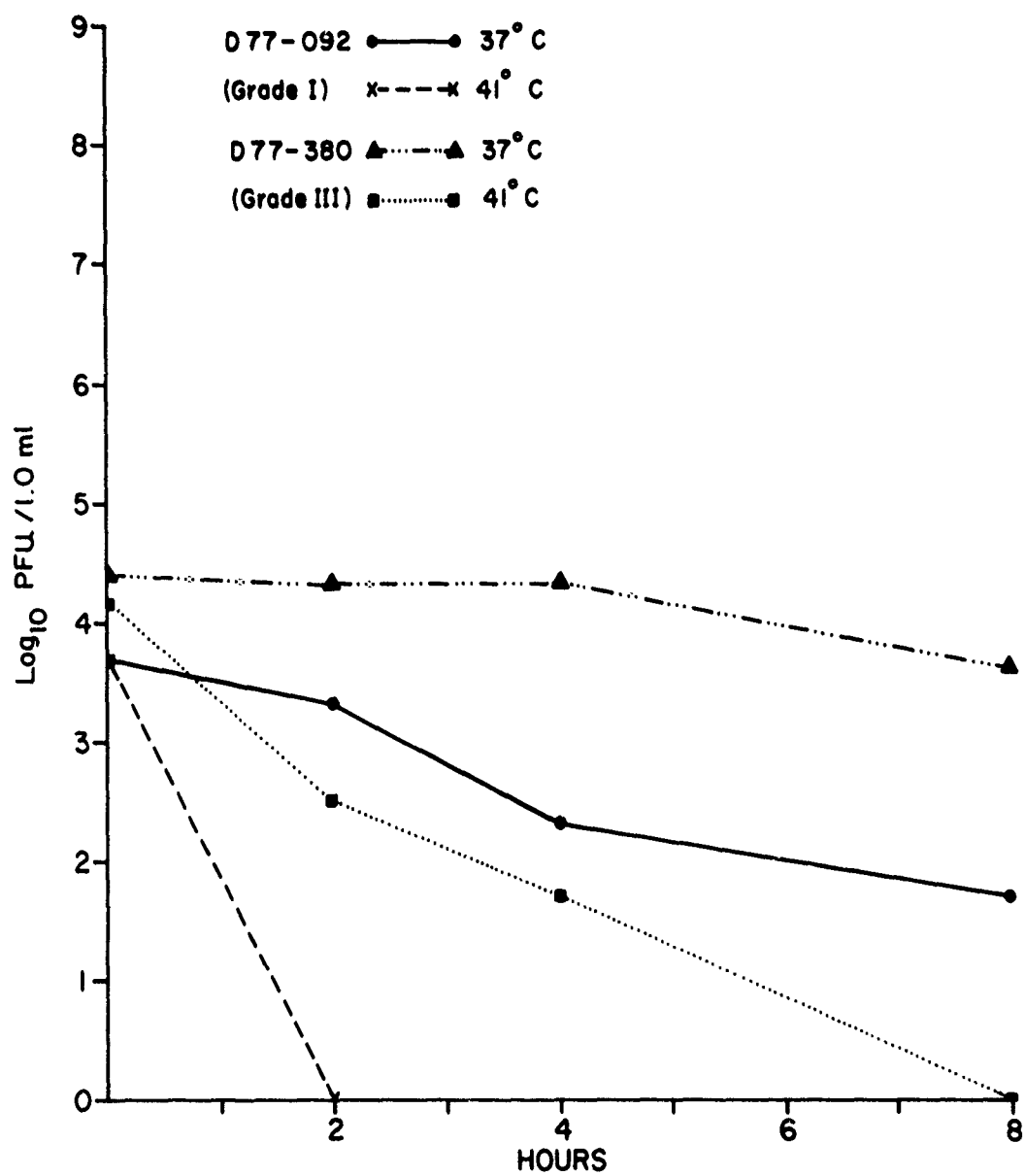


Figure 52 Thermolability of dengue virus type 4 strains.

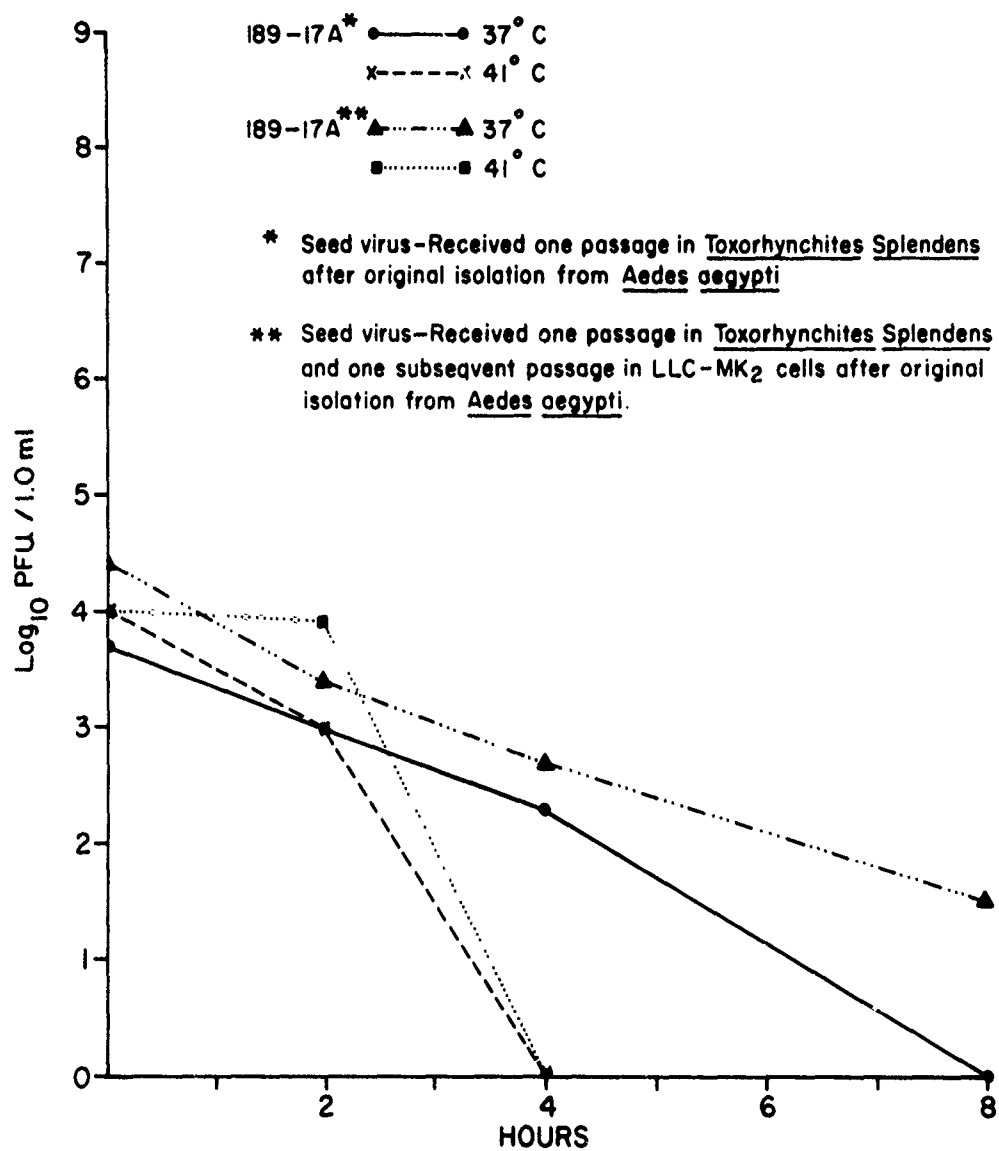


Figure 54 Thermolability of dengue virus type 2 (189-17A) that was isolated from Aedes aegypti.

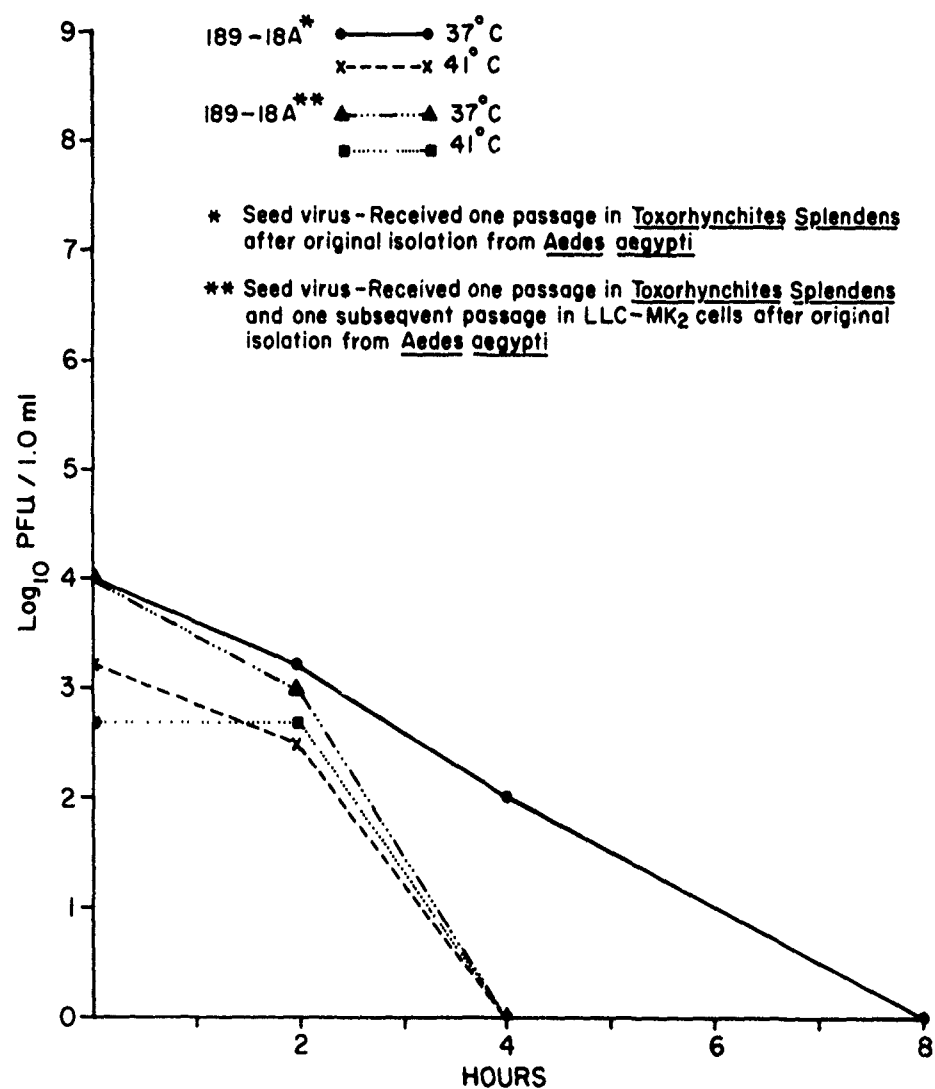


Figure 55 Thermal inactivation of dengue virus type 2 (189-18A) that originated from Aedes aegypti

was conducted to compare human serum with RPMI 1640 medium, 10% FCS, for use in thermo-stability studies of dengue viruses. The results presented in Figure 49 showed the rate and pattern of inactivation of dengue virus type 2 to be approximately the same for the two media. On the basis of these results, RPMI 1640 medium was selected for thermo-stability studies. As shown in Figures 50 and 51, the infectivity of each strain of dengue virus types 1 and 3 had reached undetectable levels after 4 hours of incubation at 37 and 41°C. No apparent difference was observed in the rate of loss of infectivity for the grade I and grade IV strains at different temperatures. In contrast, the dengue virus types 2 and 4 strains varied considerably in the rate of thermal inactivation, (Figures 52 and 53). These viruses were more thermo-stable than dengue viruses types 1 and 3, and the grade III and IV strains of dengue-2 and dengue-4 respectively, were more stable than the grades I strains of the latter dengue virus serotypes.

The results of thermal inactivation studies of dengue virus types 2 that originated from field collected *Ae. aegypti* are presented in Figures 54 and 55. The infectivity of both dengue virus strains was rapidly inactivated at 37°C and 41°C. The rate of decrease in infectivity was approximately the same for virus stocks that were subsequently propagated in LLC-Mk2 cells.

The yield of dengue virus type-2 that was obtained from plasma and cellular component of blood of patients with different grades of illness is presented in Table 86. No apparent relation was observed between the yield of virus and the grade of illness of the patient. The yield of virus varied according to temperature and the type of specimen. Although the viruses replicated at different temperature, only strain D78-135 appeared to be temperature sensitive. The distribution of viruses according to source and temperature permissive for replication is presented in Table 87.

27. Prevalence of Anti-Dengue HAI Antibody in Bangkok Infants

OBJECTIVES:

1. To establish the age-specific prevalence of anti-dengue antibody in Bangkok infants.
2. To estimate the rate of disappearance of passively acquired maternal anti-dengue antibodies.
3. To estimate the prevalence of actively acquired anti-dengue antibody in Bangkok infants.

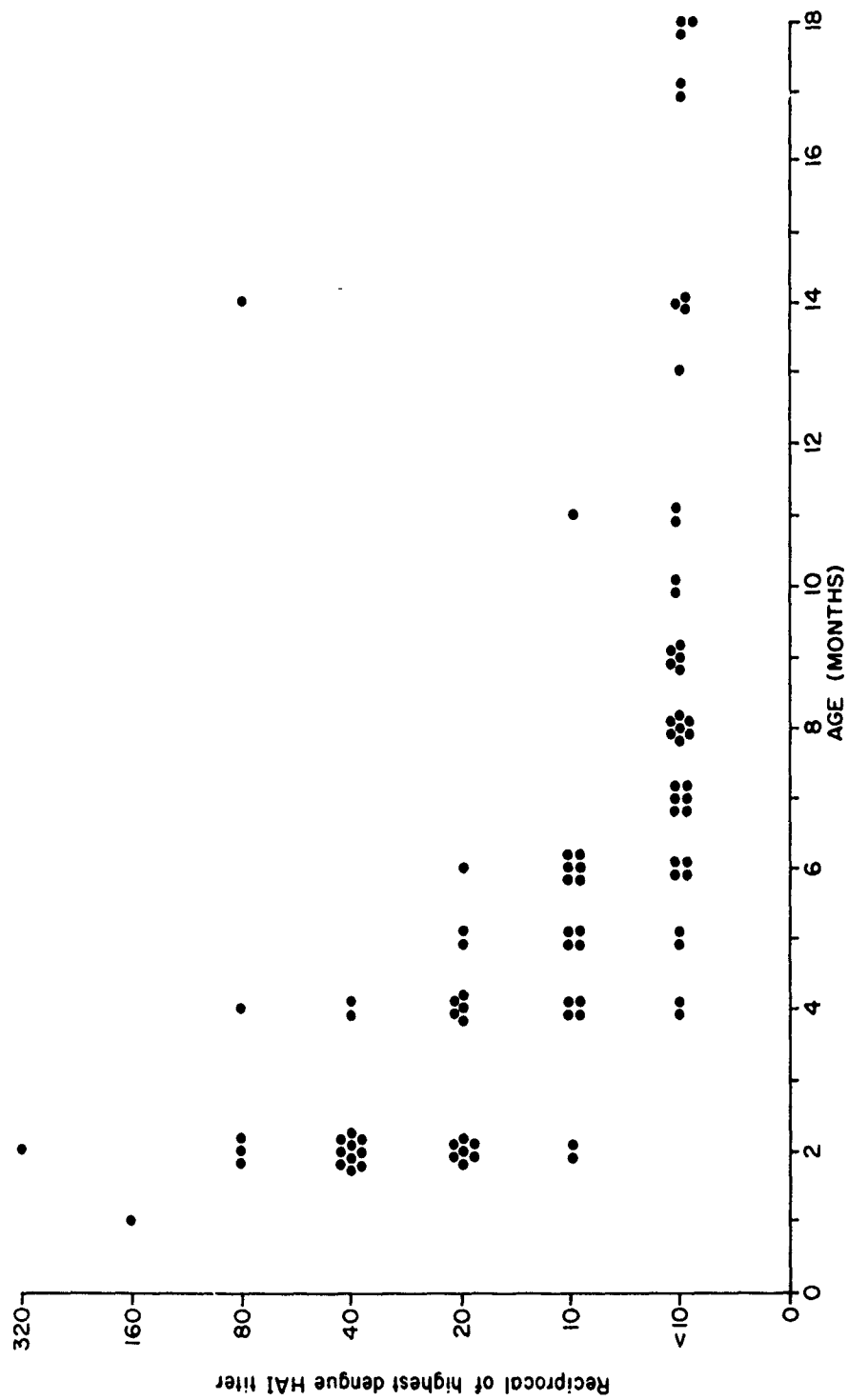


Figure 56. Graph of Dengue HAI Antibody Versus Age in Specimens from Healthy Bangkok Infants

BACKGROUND: The age-specific attack rate of dengue hemorrhagic fever (DHF) is biphasic, with the main peak modal age of 5 to 6 months and a smaller peak with a modal age of 7-12 months. Halstead's demonstration of enhanced dengue virus growth in leukocytes by very low levels of homologous or heterologous anti-flavivirus antibody has led to an examination of the role of passively acquired maternal antibody in DHF in infants (144). This study was undertaken to accurately estimate the decay kinetics of maternal anti-flavivirus antibody in Bangkok infants.

METHODS: As part of a study on the prevalence of anemia in normal children, infants attending a well baby clinic at Bangkok Children's Hospital on Wednesday afternoons during June through August 1979 were subjected to finger lancing and blood collection. Three to five 70 microliter capacity capillary hematocrit tubes were collected from each patient, and after hematocrits were determined by centrifugation, the tubes were scored and broken just above the buffy coat to retrieve the serum layer. Twenty-five microliters of serum was then acetone extracted and assayed for HAI activity against D1, D2, D3, and D4 antigens by the standard AFRIMS micro-titer technique.

RESULTS: Twenty-four of 24 (100%) of infants two months old or younger had detectable HAI to at least one dengue virus type antigen. The proportion of antibody positive infants steadily declined until age 7 months; none of 22 infants between 7 and 10 months old had detectable antibody. Two of 14 (14%) of infants 11 to 18 months old had antibody; in these two children the antibody was probably acquired actively through infection rather than transplacentally. (Figure 56)

This study will continue until sera from approximately 500 children have been tested. Other antibody determinations (e.g. anti-HAV) are planned for these sera.

28. Effect of Intravenous Inoculation of Bordetella Pertussis Vaccine on the In vivo Viremia and Antibody Response to Flavivirus Infection in Rhesus Monkeys

OBJECTIVES:

1. To determine if intravenous inoculation of Bordetella pertussis vaccine "primes" Rhesus monkeys to develop exaggerated viremias and antibody responses when the animals are subsequently inoculated with infectious flaviviruses.

2. To compare the antibody response of the "pertussis primed" monkeys to that of normal uninoculated control animals and control animals experiencing a secondary type antibody response.

BACKGROUND: Typically Rhesus monkeys develop very mild infections when inoculated with wild or prototype dengue (D) and Japanese encephalitis (JEV) strains, with no observable signs or symptoms and low level viremias (1-2 logs).

Whitehead et al. (145) reported preliminary observations of increased viremia in gibbons inoculated intravenously with Bordetella vaccine; similar preliminary observations have been made in rhesus monkeys (Halstead, personal communication).

We reasoned that if in fact viremia levels could be augmented by prechallenge pertussis inoculation, this might provide a more rigorous model for vaccine efficiency testing.

Virus typing in the Department of Virology by the plaque reduction neutralization (PRNT) method for the past decade has employed monkey anti-sera raised against local Southeast Asian strains of dengue viruses. Although these sera have been used with great effectiveness, reference sera raised against the prototype strains of virus could be valuable for periodic validation of the typing technique. The high mouse passage prototype strains in this laboratory often provoke only a weak HI and neutralizing antibody response. As a separate control group for the pertussis primed monkeys, we also investigated the antibody response of monkeys on rechallenge with homologous prototype virus six months after original challenge.

METHODS:

Rhesus monkeys: The rhesus monkeys challenged were divided into three groups (1) Normals: no previous flavivirus exposure, no pertussis inoculation (2) Immunes: previous inoculation with homologous prototype dengue or JEV prototype virus six months previously, no pertussis inoculation (3) Pertussis inoculated: no previous flavivirus exposure; 2.0 ml of pertussis vaccine intravenously on day-1 and 2.0 ml intravenously on day 2.

All normal and pertussis inoculated monkeys were shown to be antibody free for dengue-1-4 and JEV by HAI and PRNT 50 before the start of the experiment. The antibody response of the "immunes" to original challenge six months previously is shown in Table 88.

(

Viruses: Dengue 1 (Hawaii) SM16 (2×10^6 PFU/.1 ml); Dengue 2 (New Guinea C) SM30 (2×10^6 PFU/.1 ml); Dengue 3 (H87) SM26 (9×10^6 PFU/.1 ml); Dengue 4 (H241) SM32 (8×10^7 PFU/.1 ml); JEV (Nakayama) SM13 (2×10^8 PFU/.1 ml). One ml of a 20% fresh mouse brain preparation of each virus strains in normal saline was inoculated into monkeys intravenously.

Vaccine: Plain Bordetella pertussis vaccine, 20×10^6 /0.5 ml, lot 25 April 1979 (courtesy of Dr. Nathirat Sankavipa, Director, Virus Institute, Department of Medical Science, Ministry of Public Health).

Blood samples: Three ml of heparinized blood was obtained from each animal on days 0, 1, 2, 4, 6, 8, and 10 for determination of complete blood count and virus isolation studies. Blood without heparin for serologic studies was obtained on the following schedule: 10 ml on days -1, 0, 6, and 10; 25 ml on days 15 and 20; 30 ml on day 30, 35, and 59.

Viremia assays: A 1:1 mixture of plasma with buffy coat was assayed for virus by the direct and delayed plaque techniques in LLC-Mk2 cells and by the mosquito inoculation technique (Toxorhynchites). Detection of virus in inoculated mosquitoes was performed by examination of head squash preparations by direct fluorescence and by inoculation of thorax-abdomen suspensions into LLC-Mk2 cells for both direct and delayed plaque assays.

Serology: Antibody responses were measured against D1-4 and JEV by a standard laboratory microtiter adaptation of the HAI method of Clark and Cassals, and by microtiter adaptation of the plaque reduction neutralization test on LLC-Mk2 cells.

RESULTS: Hematology: The normal and immune monkeys developed a polymorphonuclear leukocytopenia on day 2 post infection; 4 of 5 monkeys in each group remained granulocytopenic for the duration of the study. The pertussis-inoculated group developed a prominent granulocytosis on day 0; although the total granulocyte counts fell in these monkeys, they never dropped below the preinoculation levels. Total lymphocyte counts remained unchanged in the normal and immune groups; in the pertussis inoculated group a relative lymphocytosis appeared on day 6, was prominent on day 8, and returned to normal on day 10. Results are presented in Figure 57

Viremia: Results of buffy coat-plasma mixture assays for virus are presented in Table 88. Overall viremia was detected in 10 of the 15 study monkeys: 1 of 5 immunes, 4 of 5 normals, and 5 of

Table 88 Viremia and Antibody Response Following Primary Inoculation of Indian Rhesus Monkeys with Prototype Dengue and JE Viruses.

Monkey No.	Inoculation c	Viremia	HI/NT					
			Day after vs Inoc.	D1	vs D2	vs D3	vs D4	vs JE
G-24/	Den-1(Hawaii) SM16(2x10 ⁶ PFU)	-	15	<10	<10	<10	<10	<10
			30	<10/<10	<10	<10	<10	<10
G-327	Den-2(new Guinea- C), SM 30(1.7x10 ⁶ PFU)		15	<10	40	20	40	<10
			30	<10	40/40	20	40	<10
G-336	Den-3(H87) SM26(3x10 ⁶ PFU)	-	15	<10	<10	<10	<10	<10
			30	<10	10	20/<10	20	<10
G-337	Den-4(H241) SM32(8.5x10 ⁶ PFU)	-	15	<10	<10	<10	20	<10
			30	<10	<10	<10	10/<10	<10
G-342	JEV(Nak) SM13(9x10 ⁷ PFU)	-	15	<10	<10	<10	<10	<10
			30	<10	<10	<10	<10/<10	<10

Table 89 Detection of Viremia in Experimental Rhesus Monkeys.
MF=Virus Detected by Mosquito Inoculation with
Fluorescent Staining of Head Squash Preparation;
MP=Virus Detected by Mosquito Inoculation with
Inoculation of Thorax-Abdomen Suspension onto LLC-Mk2
Cells and Observing for Plaques; D=Virus Detected by
the Delayed Plaque Technique on LLC-Mk2 Cells.

Monkey Number	Virus Inoculated	Monkey History	Day post inoculation				
			2	4	6	8	10
G241	D-1	prev infect	-	-	-	-	-
G246	"	pertussis	MF,MP	MF,MP	-	-	-
G220	"	normal	MF,MP	D,MF,MP	-	-	-
G327	D-2	prev infect	-	-	-	-	-
G262	"	pertussis	D,MF,MP,MP	MF,MP	-	-	-
G347	"	normal	MF,MP	D,MF,MP	-	-	-
G336	D-3	prev infect	-	-	-	-	-
G308	"	pertussis	MP	-	-	-	-
G349	"	normal	MF,MP	MP	-	-	-
G337	D-4	prev infect	-	-	-	-	-
F339	"	pertussis	MP	-	-	-	-
G310	"	normal	-	-	-	-	-
G342	JEV	prev infect	MP	-	-	-	-
G348	"	pertussis	MF,MP	D	-	-	-
G346	"	normal	MF,MP	-	-	-	-

Table 90 Results of HAI Serology. (0= < 1/10, 1= 1/10, 2= 1/20, 3= 1/40, 4= 1/80, 5= 1/160, 6= 1/320, 7= 1/640)

		HAI Antibody Response														
Virus type	Day	Normal monkey					Immune					Pertussis				
		Dengue					Dengue					Dengue				
		1	2	3	4	J	1	2	3	4	J	1	2	3	4	J
D-1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	5	3	4	5	4	0	0	0	0	0
	10	2	0	2	1	0	7	5	6	6	6	1	0	0	0	0
	15	3	1	3	3	3	6	4	5	6	5	2	0	0	0	0
	20	3	1	2	2	2	6	4	4	5	5	2	0	0	0	0
	30	3	0	0	2	2	6	4	5	6	5	2	0	0	0	0
	35	2	0	0	2	1	6	3	5	5	5	2	0	0	0	0
	60	3	1	2	2	0	5	4	5	5	4	4	0	0	0	0
D-2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6	1	3	1	2	1	1	1	3	1	2	1	0	0	1	0
	10	3	4	3	5	3	3	4	3	5	3	1	4	2	3	2
	15	3	4	4	5	4	3	4	4	5	4	1	4	4	3	3
	20	3	4	4	4	4	3	4	4	4	4	1	4	4	3	3
	30	3	4	4	4	5	3	4	4	4	5	2	4	5	4	3
	35	2	4	3	4	4	2	4	3	4	4	1	3	3	3	2
	60	3	5	4	4	3	4	5	4	5	4	2	4	2	3	2
D-3	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
	6	0	0	2	1	0	4	4	6	6	6	0	0	0	0	0
	10	1	1	4	3	2	5	4	7	6	5	1	0	4	2	1
	15	2	1	5	3	2	4	3	5	5	5	3	2	5	4	4
	20	2	1	4	3	2	4	3	5	5	4	3	2	5	4	4
	30	1	0	4	3	2	3	3	5	5	4	2	2	4	4	4
	35	1	0	3	2	1	3	3	5	4	4	2	1	3	3	3
	60	2	1	3	2	1	2	2	3	3	1	1	0	2	2	0
D-4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	4	0	3	4	5	6	5	0	0	0	2	0
	10	0	0	3	5	2	4	4	6	7	5	0	0	0	4	1
	15	1	1	3	4	3	4	4	5	6	5	1	1	3	5	3
	20	1	1	3	4	3	4	3	5	6	5	1	1	2	4	3
	30	1	1	3	4	3	4	3	5	5	4	1	0	2	4	3
	35	1	0	3	4	3	3	2	4	5	4	1	0	2	4	2
	60	1	1	2	3	1	2	3	4	4	2	1	1	1	3	0

Table 90 (Continuation)

		HAI Antibody Response														
Virus type	Day	Normal monkey					Immune					Pertussis				
		Dengue					Dengue					Dengue				
		1	2	3	4	J	1	2	3	4	J	1	2	3	4	J
JEV	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	3	4	4	5	5	7	0	0	0	0	0
	10	2	1	3	4	6	4	3	5	5	7	0	0	0	1	4
	15	2	2	5	4	6	3	3	4	4	7	1	1	2	2	5
	20	3	2	4	4	7	3	2	4	4	7	1	1	2	3	5
	30	3	2	4	4	7	3	2	3	4	6	1	0	2	2	5
	35	2	1	3	4	6	2	2	3	3	6	0	0	2	2	5
	60	2	2	3	3	5	1	2	2	2	4	1	0	1	2	4

Table 91 Plaque Reduction Neutralization Test of Monkey Sera
Against Dengue 1, 2, 3, 4 and JE by Micro-technique

Virus Type	Monkey No.	Date blood collected	PRNT Titer				
			D1	D2	D3	D4	JE
D1	G-220	30 days	590	125	19	<10	<10
		60	1000	17	25	<10	<10
D1*	G-241	30	>640	50	43	16	<10
		60	>640	84	100	25	<10
D1**	G-246	30	190	23	10	<10	<10
		60	400	<10	<10	<10	<10
D2	G-347	30	18	600	12	<10	<10
		60	62	1850	20	<10	<10
D2*	G-327	30	65	1650	70	14	10
		60	50	2400	50	10	<10
D2**	G-262	30	10	36	21	<10	<10
		60	<10	65	30	<10	<10
D3	G-349	30	<10	22	103	<10	<10
		60	10	22	500	<10	<10
D3*	G-336	30	100	35	640	14	<10
		60	100	39	240	<10	<10
D3**	G-308	30	<10	<10	78	<10	<10
		60	<10	<10	96	<10	<10
D4	G-310	30	<10	<10	<10	<10	<10
		60	<10	<10	<10	48	<10
D4*	G-337	30	10	18	28	23	<10
		60	<10	27	11	12	<10
D4**	F-339	30	<10	<10	<10	21	<10
		60	<10	<10	<10	41	<10
JEV	G-346	30	<10	<10	<10	<10	2000
		60	<10	<10	<10	<10	1000
JEV*	G-342	30	<10	<10	<10	<10	290
		60	<10	15	10	<10	570
JEV**	G-348	30	<10	<10	<10	<10	1500
		60	<10	<10	<10	<10	680

* = Previous inoculation with homologous strain

** = Pertussis immunized

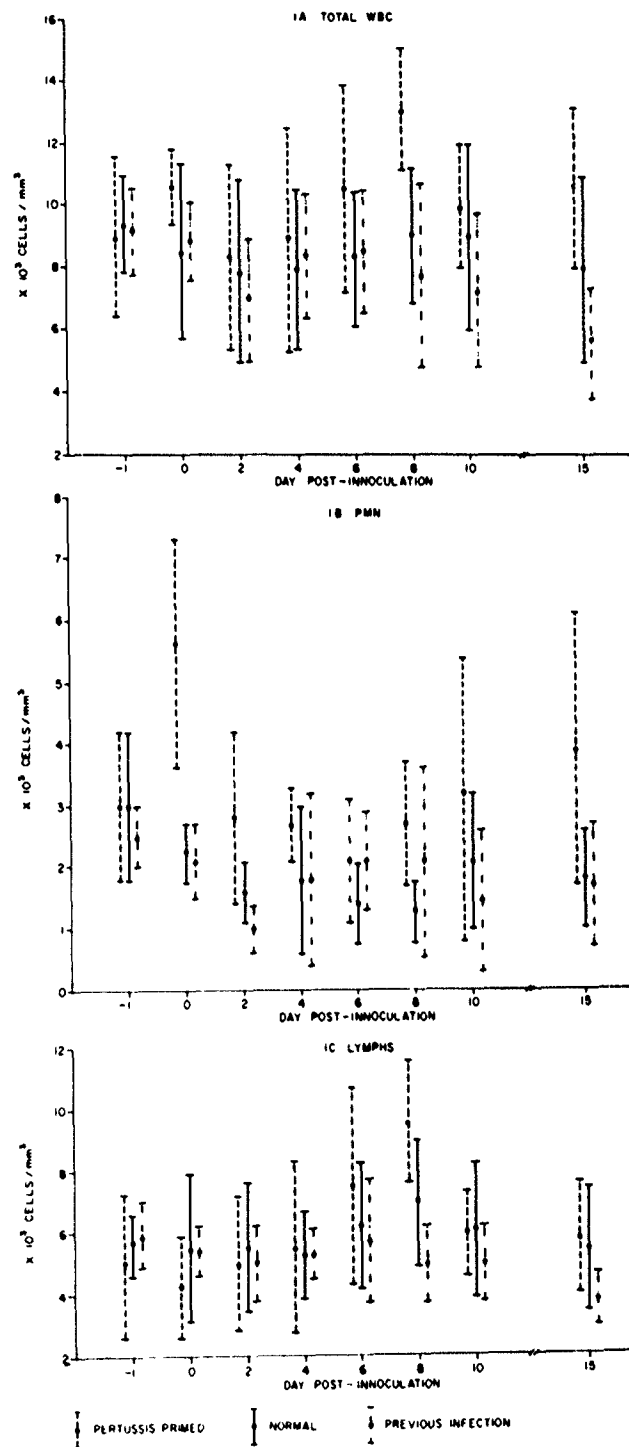
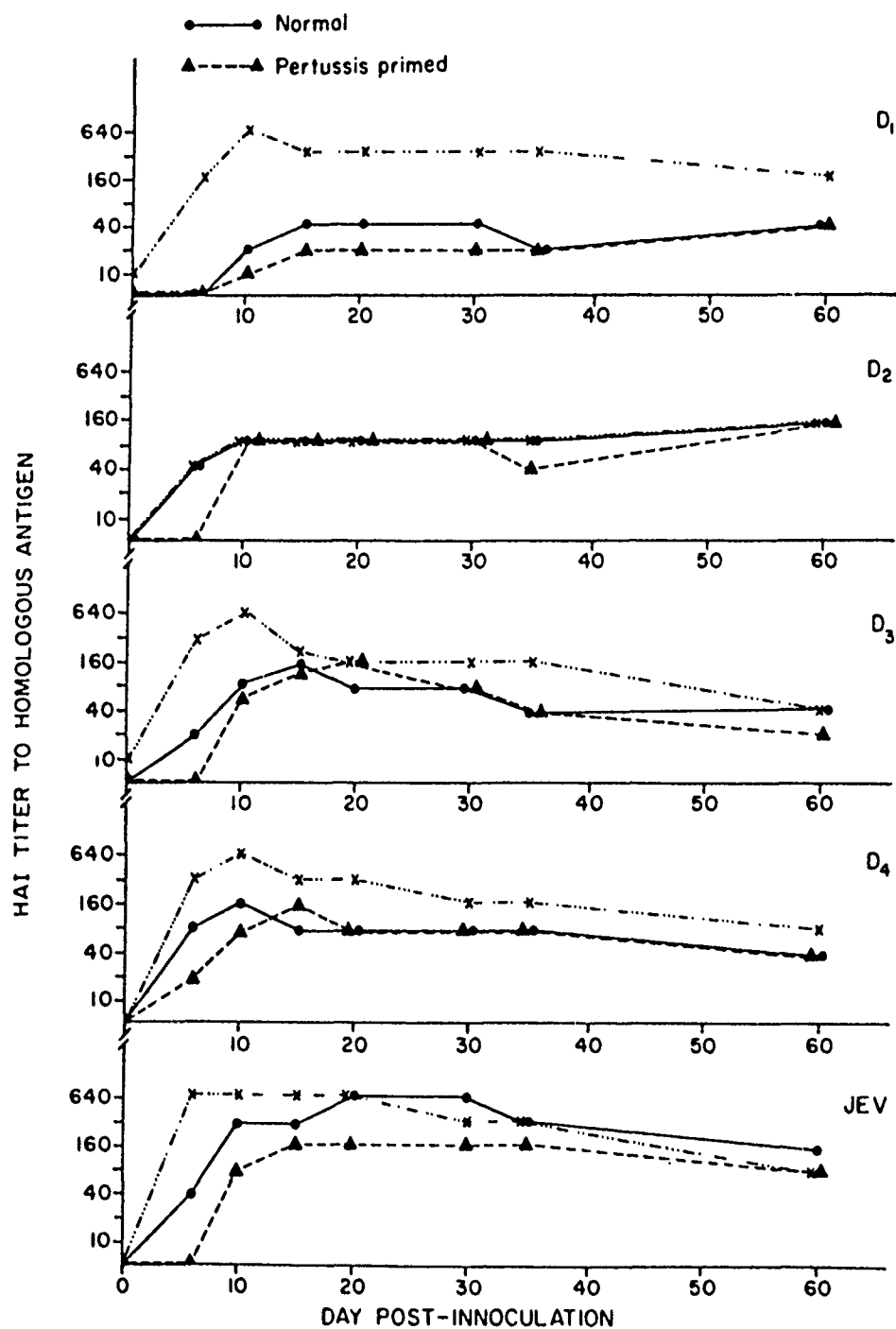


Figure 57. Leukocyte Responses in Normal Monkeys, Previously Infected Monkeys, and Pertussis Primed Monkeys Challenged with Prototype Dengue Viruses.

Figure 58 HAI Antibody Responses in Normal Monkeys, Previously Infected Monkeys, and Pertussis Primed Monkeys Challenged with Prototype Dengue Viruses.



5 pertussis inoculated. A total of 16 blood specimens yielded virus isolates; virus was detected by the plaque assay on LLC-Mk2 cells in 3, by mosquito head squash fluorescence in 11, and by assay of thorax-abdomen suspensions on LLC-cells in 15.

Antibody response: The HAI antibody responses of infected monkeys are shown in Figure 58 and Table 90. For every virus type the immune monkeys developed the most rapid and highest antibody response, the normal monkey produced a slower and somewhat lower response, and the pertussis primed monkey had the slowest and lowest response. Four of 5 normal monkeys had developed early antibody by day 6, compared to only 1 of 5 pertussis inoculated monkeys. Both the primary and secondary antibody responses of the D-2 challenged monkeys showed considerable HAI cross reactions to heterologous antigens, especially to dengue type-4.

Plaque reduction neutralization (PRNT) antibody titers are presented in Table 91. Titers were invariably highest against the homologous virus type. In every instance the normal monkey developed a greater PRNT antibody response than the corresponding pertussis immunized monkey, and the previously inoculated monkeys invariably developed the broadest antibody responses.

29. HLA Antigens of Dengue Hemorrhagic Fever Patients

OBJECTIVE: To determine if children with dengue hemorrhagic fever (DHF) have a unique distribution of HLA types.

BACKGROUND: The serological response of DHF patients against dengue antigens may be classified into primary and secondary type responses (146). The severity of the disease appears to be more pronounced in females and in secondary dengue infections (147,148). It is possible that some individuals are more susceptible to this particular infection than others. In addition, there may be some genetic markers that control the immunologic mechanisms.

The present investigation was undertaken to determine whether there were any significant deviations from the normal distribution of HLA-A and HLA-B in DHF patients.

MATERIALS AND METHODS: A retrospective study was carried out on 87 unrelated DHF patients from the case collection of Children's Hospital, Bangkok, Thailand (clinical data) and the AFRIMS (serological data). Clinical criteria for DHF followed those of Nimmannitya (149) and serological criteria those of Winter et al. (146). Eighty-seven patients (39 male and 48 female) were included in this study. There were 8 and 79 patients with primary and secondary infections, respectively.

The analysis was carried out according to sex, primary and secondary responses, and grading of the patients.

The control group consisted of 138 apparently healthy and unrelated local blood donors, 109 male and 29 female.

HLA typing was done using the standard two-stage lymphocytotoxicity technique (NIH) (150). Fifty-nine antisera from NIH were used to detect the following HLA-A and B locus specificities: A1, 2, 3, 9, 10, 11, 19, 28, 29, w23, w24, 225, w26, w30, w31, w32, w33, w34, w36; B5, 7, 8, 12, 13, 14, 15, 17, 18, 27, 40, w16, w21, w22, w35, w38, w41, w42.

RESULTS: HLA-A1 and A9 of the primary response group and HLA-A2 and B blank of secondary response groups were found to be significantly increased as compared with the normal control group ($p < 0.05$). HLA-A2 was also significantly increased in the male patients ($p < 0.05$), while HLA-B17 was significantly increased among the female patients ($p < 0.05$). A significantly increased prevalence of HLA-B blank was observed in grade 3 and among all DHF patients ($p < 0.05$). Decreased prevalences of HLA-B13 ($p < 0.05$) and HLA-B15 ($p < 0.05$) were observed in the secondary response group, and in grade two patients, respectively. However, the significance of these findings (and p values) should be open to question, since 38 antigen were tested for; it is not unlikely that the findings are due to chance.

30. Suppression of Lymphocyte Blastogenic Response by Sera from Patients with Dengue Hemorrhagic Fever (DHF)

OBJECTIVE: To investigate the suppressive effect that sera from DHF patients has on the response of lymphocytes stimulated with plant lectins.

BACKGROUND: Sera from patients with various infections (23, 151) have been shown to suppress the responsiveness of lymphocyte populations stimulated with plant lectins. This may be an immune regulatory mechanism influencing the interaction of specific lymphocyte subpopulations (151) or may be a non-specific response to infectious agents.

In the present preliminary study we are investigating the effect that sera from DHF patients has on the responsiveness of normal lymphocytes to stimulation with plant lectins.

METHODS: Acute and convalescent sera from children clinically diagnosed as having dengue hemorrhagic fever was obtained from Bangkok Children's Hospital. Aliquots of each sample were frozen

Table 92 Inhibitory Action of DHF Sera on Mitogen Response of Human Lymphocytes.

Mitogen	Pooled Serum ¹		
	Normal	Acute (day 0) (DHF)	Convalescent (day 14) (DHF)
PHA	156.43 ²	56.67	170.59
Con A	166.94	73.52	135.25
PWM	116.06	44.16	137.71

1. Each serum pool from 5 individuals
2. Stimulation index when pooled sera is added to wells (20% v/v)

(-70°C) for future serological confirmation. The mitogen inhibition assay was performed as previously described (23).

RESULTS: Table 92 shows that pooled acute sera from DHF patients inhibits the responsiveness of normal lymphocytes to mitogen stimulation, while convalescent sera does not. Additional studies are being conducted to confirm these results and to determine the effect that sera from DHF patients has on normal, autologous and allogenic T cells and B cells. This is a preliminary report.

31. Lymphocytotoxins in Serum from Patients with Dengue Hemorrhagic Fever (DHF)

OBJECTIVE: To identify lymphocytotoxic activity in the sera of Thai children with dengue hemorrhagic fever.

BACKGROUND: Serum lymphocytotoxins have been reported in a wide variety of disease states (152). It is not clear why such factors are elicited and very little is known concerning the action of naturally occurring lymphocytotoxins in the immune response to infectious agents. Earlier work in this Laboratory (153) indicated that the percentage of peripheral T-cells in patients during the acute stage of DHF is reduced when compared with the percentage of T-cells in peripheral blood during the convalescent stage. Lymphocytotoxic factors directed against specific cell populations may be one mechanism responsible for the decrease in the number of circulating lymphocytes and thereby have a regulatory influence on the immune response of individuals to dengue hemorrhagic fever. Therefore, we are presently screening sera from patients with DHF to determine the incidence of lymphocytotoxins, their temperature of optimal activity, and biological effect on autologous and allogenic lymphocytes.

METHODS: Cytotoxic assays are performed with acute sera from patients with dengue hemorrhagic fever following the previously described (152) modification of Terasaki's methodology (153).

RESULTS: Table 92 summarized the results, to date, of lymphocytotoxin assays performed with sera from patients with DHF and lymphocytes from uninfected controls. Killing due to control sera averaged only 4.5% (37°C) and 6.1% (15°C).

Additional studies are being performed in this area in order to determine the in vivo relevance of antilymphocytotoxins in patients with dengue hemorrhagic fever.

Table 93 Lymphocytotoxins in Acute Dengue Hemorrhagic Fever

% Cytotoxicity of Normal Adult Allogenic Lymphocytes

<u>Sample</u>	<u>Temperature</u>	
	<u>37°C</u>	<u>15°C</u>
1	8.5*	23.5
2	4.5	4.5
3	4.0	6.0
4	7.0	9.0
5	7.0	42.0
6	7.0	24.0
7	6.5	27.5
8	7.0	8.0
9	5.5	12.5
10	8.5	10.0
11	8.0	51.0
12	7.5	35.5

*Percent: Cytotoxicity by dye exclusion

32. Detection of Infectious Virus and Viral Antigens in
Tissue Specimens from Fatal Cases of DHF

OBJECTIVE: To detect viruses and viral antigens in tissue specimens from fatal cases of dengue hemorrhagic fever (DHF) and to identify the infected cell types.

BACKGROUND: Dengue viruses are rarely recovered from autopsy materials from patients dying of DHF. In 1964 SEATO Lab workers were able to isolate dengue virus from tissues of only two of 169 fatal cases of DHF examined (154). However, techniques for isolation of dengue viruses from clinical materials have improved considerably since earlier studies.

A body of evidence is growing to suggest that dengue viruses replicate, in primates primarily within the reticuloendothelial system, predominantly within macrophages or other phagocytic cells. Virus and viral antigens have been detected circulating in the blood in a variety of mononuclear cells, but to date identification of viral antigen in human tissues has not been achieved, and the cell types involved have not been identified.

METHODS: Fatal Cases: Four fatal cases of DHF at Children's Hospital and Ramathibodi Hospital during 1978 were studied (Table 94). In three of the four cases the clinical diagnosis was DHF, and in one the clinical diagnosis was unclear, with Reye's syndrome and DHF being considered. The age distribution of fatal cases was quite different from the age distribution of non-fatal cases seen during 1978 at Children's Hospital; three cases were in infants less than one year old and one was in a 17 year old.

Case 477-78, on the basis of a high fixed hemagglutination inhibition (HI) antibody titer, was serologically assigned a presumptive diagnosis of DHF. In case 78-110 a clear four fold HAI titer rise was not observed, but the detection of anti-dengue-2 IgM antibodies by sucrose density gradient fractionation proved the occurrence of a recent dengue infection. No serum was obtained from case 318-78; the anti-dengue HAI titer in case 319-78 was too low to permit sucrose density fractionation for detection of IgM.

In cases 318-78, 319-78, and 477-78, autopsy findings were consistent with dengue hemorrhagic fever, with diffuse cutaneous and visceral petechia and hemorrhages pericardial and pleural effusions, and ascites. In addition, in case 477-78, jaundice was present and the liver showed "central necrosis;" a test for HBsAg

Table 101 Evaluation of HAVAB Comparative Results With Other
Anti-HAV Assays

Serum or plasma source	AFRIMS HAVAB(R) Log ₁₀ titer	Purcell NIH-IAHA Log ₁₀ titer	WRAIR SPRIA %Block	Baylor SPRIA %Block
Chimp 173 pre	<1.3	1.0	0	-
Chimp 267 pre	<1.3	1.0	0	-
Ex 353	<1.3	-	0	0
Ex 272	<1.3	-	0	18
Ex 337	<1.3	-	0	36
Chimp 173 post	3.7	3.3	74	-
Chimp 267 post	3.7	3.6	69	-
Smith (AK 003)	4.9	4.2	74	98
LEE (AK 005)	4.2	4.3	71	-
Bennett (AK 059)	3.9	3.6	80	98
HYLTON (AK 019)	4.5	3.6	71	-
WHO Ref A	4.8		4.8*	
B	4.3		4.5*	
C	3.4		3.1*	
D	4.3		4.2*	
E	4.2		4.6*	
F	5.5		4.5*	
LOCAL ISG				
Hyland	4.6			
Metabolic, Inc.	4.6			

* Log₁₀ of reciprocal dilution of 50% blocking end point

Figure 64 Graph of Counts per minute versus Dilution of Chimpanzee or Human Plasma or Serum in HAVAB (R)

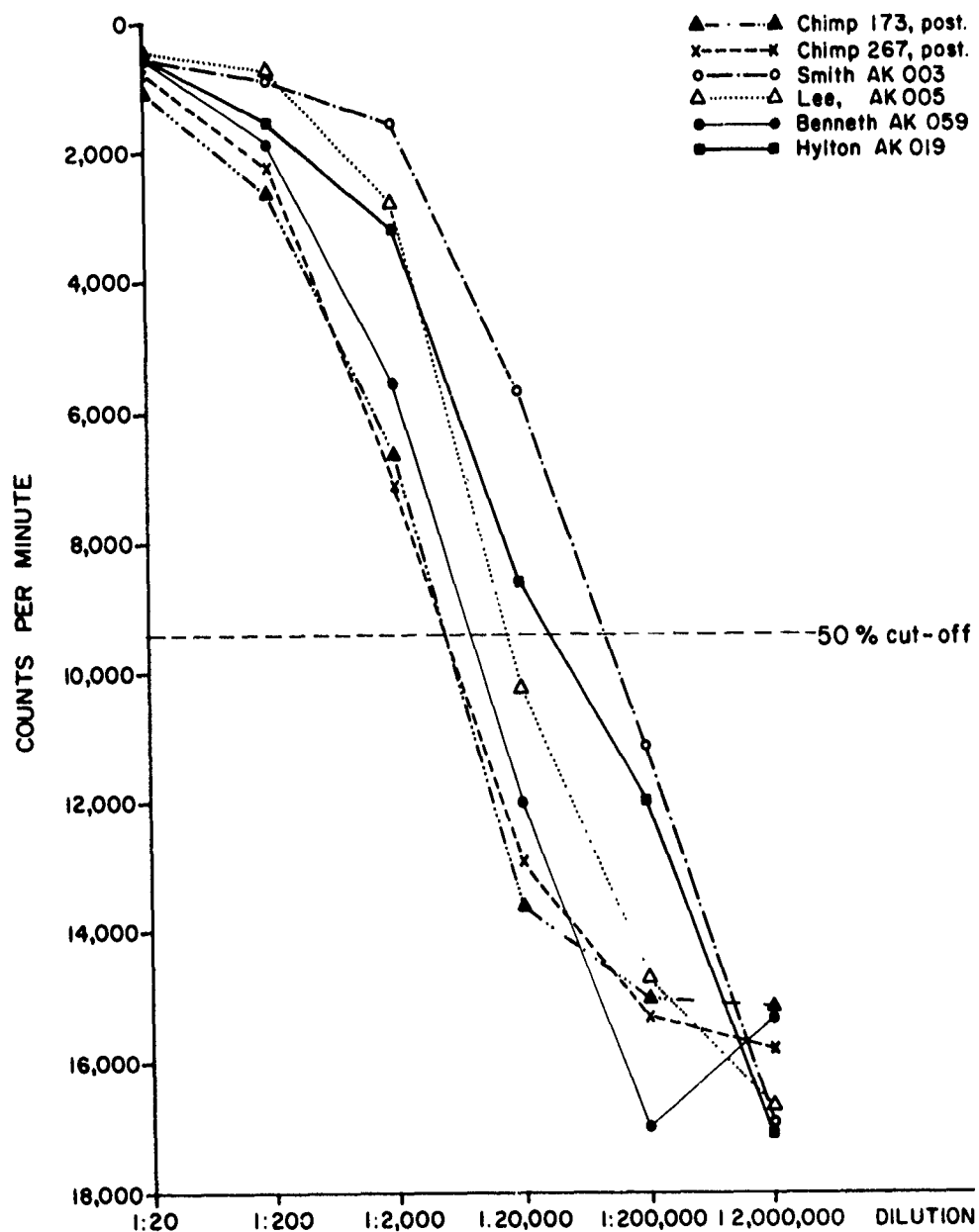
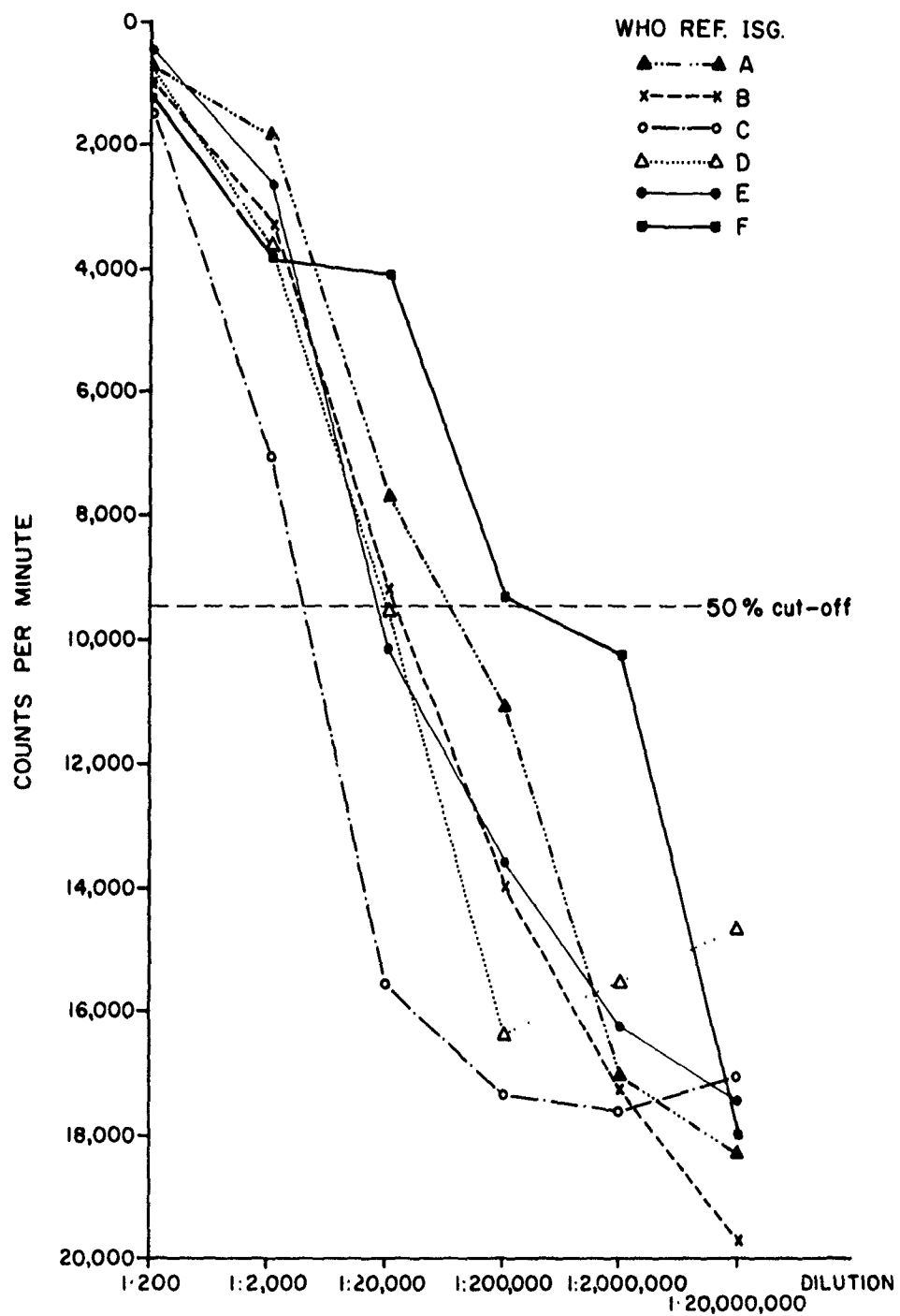


Figure 65 Graph of Counts per minute versus Dilution of Immune Serum Globulin Specimens in HAVAB (R)



METHODS: A battery of 17 serum, plasma, and globulin specimens were provided by MAJ Stanley Lemon, Department of Virus Diseases, Walter Reed Army Institute of Research (WRAIR). Characteristics of the specimens are presented in Table 100. In addition, samples of two lots of immune serum globulin were obtained from the Health Clinic at the U.S. Embassy in Thailand (Hyland ISG, Lot # 0632#002AA; and Metabolic Incorporated ISG Lot M 8324). The HAVAB (R) assay (a blocking solid phase radioimmuno assay) was performed according to the Manufacturer's recommendation without modification.

RESULTS: Graphs of the raw counts per minute (CPM) obtained with serial ten-fold dilutions of positive specimens are shown in Figures 64 and 65. Note that in the HAVAB system the lowest dilution of serum (or plasma) which may be tested is 1:20. The titer of a specimen was determined as that point where the dilution versus CPM curve intersected the cut-off value.

The \log_{10} of the reciprocal of the HAVAB (R) titers are presented in Table 101 along with similar data obtained by other laboratories using different assays on the same specimens. (Data provided by MAJ Lemon).

37. Age-Specific Prevalence of Anti-Hepatitis A Virus Antibody in Thailand

OBJECTIVE: To determine the age-specific prevalence of anti-HAV antibodies in healthy Thais.

INTRODUCTION: In the few developing countries thus far studied, most life-long residents become infected with the hepatitis A virus (HAV) before reaching adulthood (162, 163). We report here our observations on the age-specific prevalence of antibody to hepatitis A (Anti-HAV) in three geographically defined populations of healthy Thais.

METHODS: Study Populations: Three populations were studied, each of which had previously been the subject of epidemiologic studies by this Institute.

1. Huay Khwang District (Bangkok): In 1971-73, random households in a low income housing project were selected for investigations of the epidemiology of hepatitis B virus infections (164). Of the 687 original subjects, serum specimens were available from 569 (83% of the original population).

From a list of subjects with available specimens, subjects were stratified by age and a sample was chosen at random so that approximately 20 sera were tested for each two year age group less than 15 and each five year age group over 15. The total number of specimens tested was 308. Ninety percent of the specimens tested were drawn from blood sample taken between 20 July and 10 October 1971 and 10% were from samples taken between 10 April and 9 June 1972.

2. Ban Tablan: In late 1976, an attempt was made to obtain blood specimens from all of the residents of a relatively isolated rural village approximately 250 km north west of Bangkok, again for studies of hepatitis B virus (165). Of the total population of 1,014, blood specimens were obtained from 774 individuals (76% of the population). From these sera, specimens were chosen at random to obtain approximately 15-20 specimens for testing from each age bracket. The total number of specimens tested was 206.

3. Phibunprachasan School, Din Daeng District (Bangkok): In 1977, a survey of togavirus and hepatitis B virus infections was made at a lower-middle class public school in Bangkok (23). Blood specimens were obtained from 1,977 children, 98% of the school population. From each 2-year age group, approximately 40 sera were chosen at random for testing. The total number of specimens tested was 232. All serum specimens had been preserved frozen at -20°C until tested.

Anti-HAV testing: A commercial solid phase competition I125 radioimmunoassay kit was used (HAVAB (R), Abbott Laboratories) in accordance with the Manufacturer's recommendations. For titering, serial 10 fold dilutions of serum were made in phosphate buffered saline without added protein.

Anti-HBs and HBsAg testing: Sera were assayed for evidence of previous hepatitis B virus infection with commercial kits (AUSAB (R) and AUSRIA-II (R), Abbott Laboratories).

Poliovirus neutralizing antibody testing: Sera from the Huay Khwang and Phibunprachasan collection were tested for neutralization of 100-300 TCID₅₀ prototype poliovirus types 1, 2 and 3 in Hela cells. Dilutions of sera and virus were incubated together for 30 minutes at room temperature in microtiter plates. Cell suspensions were inoculated into the wells with the serum-virus mixture and covered with a layer of mineral oil. Plates were incubated at 37°C and endpoints were read by the metabolic inhibition method on days five to seven. Sera with titers of 1:5

Figure 66 Age Specific Prevalence of Anti-HAV in Three Populations of Healthy Thais.

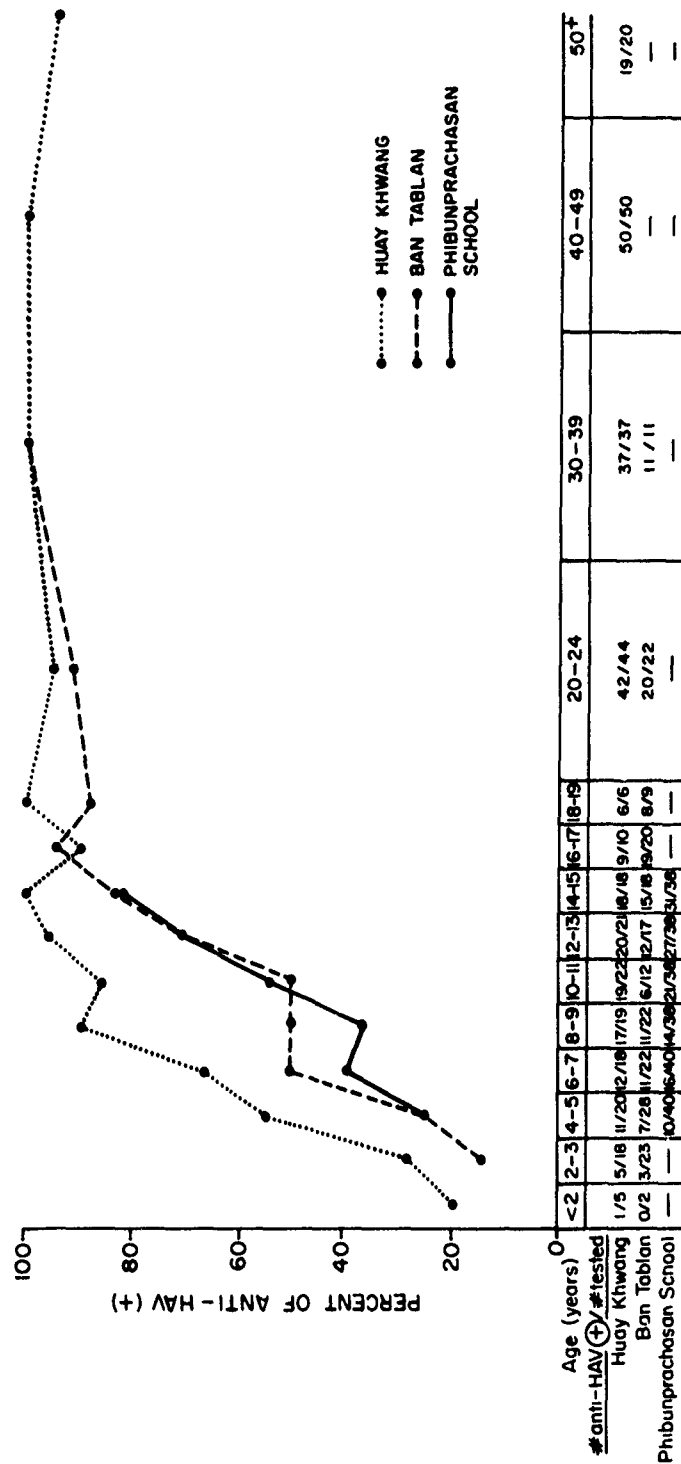


TABLE 103
PREVALENCE OF ANTI-HAV IN VARIOUS AGE GROUPS OF CHILDREN IN RELATION
TO POLIOMYELITIS VIRUS AND HEPATITIS B VIRUS EXPOSURE STATUS

	Age (years)							
	2-3		4-5		6-7		8-9	
	No. tested	No. positive (%)	No. tested	No. positive (%)	No. tested	No. positive (%)	No. tested	No. positive (%)
<u>Poliomyelitis virus exposure status</u>								
Polio positive ^a	4	2(50)	36	12(33)	36	18(50)	34	19(56)
Polio negative ^b	14	3(21)	23	9(39)	20	9(45)	20	10(50)
<u>HBV exposure status</u>								
HBV positive ^c	9	2(22)	13	6(46)	14	7(50)	8	6(75)
HBV negative	30	5(17)	35	12(34)	27	16(59)	33	22(67)

- a. Serum polio neutralizing antibody titer $\geq 1:5$ for all three polio types.
b. Serum polio neutralizing antibody titer $< 1:5$ for a least one polio type
c. Either HBsAg or anti-HBs detectable in serum.

were interpreted as negative for antibody (166).

RESULTS: Age specific prevalence of anti-HAV antibody: The prevalence of anti-HAV serum antibody in the three populations studied is presented in Figure 66. The age at which approximately 50% prevalence was attained was four to five years for Huay Khwang residents, 8 to 9 years for Ban Tablan village residents, and 10 to 11 years for Phibunprachasan school children. After age 15, the antibody prevalence approached 100% in all three populations. Antibody prevalence, or more specifically, the prevalence of the lack of antibody did not appear to be either family or household associated. In the three populations, there was no apparent difference in the age-specific antibody prevalence between males and females.

Anti-HAV antibody in adults and children: Ninety-four percent of adults (16 years of age or older) tested from Ban Tablan were antibody positive (51/54), as were 98% (159/163) of adults from the Huay Khwang district. Determination of the anti-HAV antibody titer of all positive serum specimens from young children (less than eight years old) and 12 positive specimens from adults (more than 40 years old) from Huay Khwang showed geometric mean titers (mean + S.D.) of 1:700 + 130 for children and 1:240 + 70 for adults.

Anti-HAV prevalence and anti-poliomyelitis virus prevalence: Among five year olds in the Huay Khwang population, the percent of children with neutralizing antibodies to polio types 1, 2 and 3 were 74%, 95%, and 80% respectively, and among five year olds at the Phibunprachasan School the comparable percentages were 82%, 87% and 84%. Table 102 compares the age-specific prevalence of anti-HAV for children with antibody to all three poliovirus types with that for children lacking antibody to one or more poliovirus types. There is no detectable difference.

Anti-HAV prevalence and HBV marker prevalence: Table 102 presents the prevalence of anti-HAV in relation to previous exposure to hepatitis B virus (HBV) for children two to nine years of age in the Huay Khwang and Ban Tablan populations. There is no difference in prevalence of anti-HAV between those with previous exposure to HBV and those without exposure.

38. Susceptibility of Gibbons, Rhesus Monkeys, and Cynomologous Monkeys to Infections with the Hepatitis A Virus (HAV)

OBJECTIVES:

1. To determine if gibbons, rhesus monkeys, and cynomologous monkeys have naturally acquired circulating serum antibodies to human HAV.

2. To determine which if any of these species could be used as an experimental model for HAV infection.

BACKGROUND: Anti-HAV activity has been detected in serum specimens from several primate species. Miller et al. (167) detected anti-HAV by the immune adherence method in 14 of 23 chimpanzees, 9 of 40 grivets, 1 of 17 rhesus, 0 of 6 baboons, 0 of 1 gibbons, and 0 of 82 marmosets. Purcell et al. (168) detected anti-HAV by the immune electron-microscopy method in 8 of 8 jungle-caught chimpanzees, 0 of 16 colony-born chimpanzees, 4 of 12 rhesus, 4 of 4 patas monkeys, 1 of 5 African green monkeys, 0 of 4 baboons, and 0 of 14 coebus monkeys. Before the development of serologic tests for HAV, it had been determined that only marmosets and chimpanzees were good models of acute infection with HAV (169).

The finding of antibody to HAV in other primate species suggests that perhaps these species also are susceptible to infection.

METHODS: Serum specimens were obtained from 6 gibbons, 10 cynomologous monkeys, and 92 rhesus monkeys caged at the AFRIMS Veterinary Medicine facility. The history of each primate was reviewed to determine if the animal was: (1) colony born or wild captured (2) individually caged or gang caged. Specimens were tested for anti-HAV by the HAVAB (R) solid phase competition radioimmunoassay. The sera of two rhesus monkeys was precipitated by addition of ammonium sulfate to 33% saturation, the precipitate resuspended in PBC and dialyzed against PBS.

RESULTS: Results of anti-HAV screening of AFRIMS primate sera are presented in Table 103. Overall 2/6 (33%) of gibbons, 6/10 (60%) of cynomologous monkeys, and 13/92 (14%) of rhesus monkeys had detectable anti-HAV activity in their sera. Mean % blocking for positive specimens for each species were 76%, 95%, and 87% respectively, well above the cut-off value of the test of 50%. Ammonium sulfate precipitation of two rhesus sera showed that blocking activity was associated with the protein globulin fraction in both.

Historical data of individual antibody positive and antibody negative primates suggests (1) that gibbons and perhaps rhesus

Table 103 Anti-HAV in AFRIMS Primates - 1979

<u>Species</u>	<u>Rearing History</u>		<u>Caging History</u>		
	Colony born	Captured	Individual	Gang	Total
Gibbon	0/4	2/2	2/6	0/0	2/6(33%)
Cynomologous	5/5	1/5	0/0	6/10	6/10(60%)
Rhesus	0/0	13/92	13/92	0/0	13/92(14%)

monkeys can contract HAV in the wild, and (2) that HAV infection may have spread through the colony of gang caged cynomolgous monkeys.

Currently, challenge studies are underway in which animals of each species have been inoculated with known HAV infectious stool filtrates. These animals are being followed for the appearance of liver function test abnormalities, antigen shedding in the stool, and anti-HAV antibody response.

39. Evaluation of the Staphylococcus Protein A Absorption Method for Detecting Anti-Hepatitis A Virus Immunoglobulin M

OBJECTIVE: To evaluate the Staphylococcus Protein A absorption modification of the HAVAB (R) solid phase competition radioimmunoassay as a method for detecting anti-HAV IgM.

BACKGROUND: Establishing a laboratory diagnosis of acute infection with the hepatitis A virus is difficult. In most patients, viral excretion in the stool has ceased before the appearance of jaundice, and frequently the serum antibody titer has approached peak levels by the time the patient seeks medical care, so that demonstration of an unambiguous rise in titer may not be possible (170). Research efforts have therefore been directed toward developing assays to detect IgM antibodies with anti-HAV activity as presumptive evidence of acute infection.

At present the only widely available assay for anti-hepatitis A virus antibody is a commercial solid phase radioimmunoassay (SPRIA) based on the competition of the patient's antibody with labelled anti-HAV (competitive binding assay, CBA). Three basic methods have been proposed for pretreating sera to detect evidence of specific acute phase antibodies using the CBA method:

1. Absorb the sera with staphylococcus aureus protein A (SA absorption) to remove IgG antibodies and demonstrate residual non-absorbable activity in the absorbed sera (171).
2. Treat the whole serum with 2-mercaptoethanol (2-ME) to inactivate IgM and demonstrate a decrease in activity (172, 173).
3. Fractionate the serum by sucrose density gradient ultracentrifugation and assay the activity of the 19S and 7S fractions (174). In this study we compared the SA absorption method to the other two methods for detecting diagnostic acute phase anti-HAV antibodies in serum specimens from patients with acute hepatitis.

METHODS: Screening SPRIA on undiluted serum: Undiluted sera were screened using the HAVAB (R) as directed by the Manufacturer without modification. Greater than 50% blocking of counts was taken as evidence for specific anti-HAV antibodies in undiluted sera.

Serum specimens: Sixteen acute phase serum specimens (obtained within six weeks of the onset of symptoms) were selected from an acute hepatitis study serum bank. All serum bank specimens had previously been assayed for HBsAg (AUSRIA-II (R) Abbott Laboratories) anti-HBs (AUSAB, (R), Abbott Laboratories) total anti-HAV activity, and anti-HAV activity before and after SA absorption.

Two sera lacking anti-HAV activity by screening CBA were selected as negative controls. Fourteen sera with anti-HAV in unabsorbed sera formed the study battery: in seven the anti-HAV could be absorbed out by SA absorption (Group A) while in seven others the anti-HAV activity was not removed by SA absorption (Group N). The age and sex of the patient, day of illness, HBsAg status, % blocking with undiluted sera, and % blocking before and after absorption of a 1:15 dilution of the specimen, are presented in Table 104.

Staphylococcal protein A absorption of sera: A strain of *S. aureus* was kindly provided by Dr. Savanat Tharavanij at the Faculty of Tropical Medicine, Mahidol University. The staphylococci were grown for 24 hours in 2 litre volumes of trypticase soy broth. Whole bacteria were prepared for absorption of sera by heat inactivation and formaldehyde treatment according to the method of Kessler (175). Before absorption, all sera were diluted 1:15 in PBS pH 7.4 regardless of the percent blocking activity in the screening CBA. Absorption of an aliquot of the 1:15 dilution of serum was performed according to the method of Bradley et al. (171), except that sera were absorbed twice. Unabsorbed and absorbed aliquots were tested for percent blocking activity by the CBA method.

Sucrose density gradient fractionation of sera: Thawed serum specimens were clarified by filtration through millipore 0.45 micro filters. A 0.1 ml specimens was layered on top of a 10-40% gradient of sucrose in a 5.0 ml ultracentrifuge tube and centrifuged for 18 hours in a SW50 rotor in a Beckman L-350 ultracentrifuge at 35,000 rpm. Five drop fractions were collected by piercing the bottom of the tube. Fractions were tested by immunodiffusion against anti-human immunoglobulin (Antibodies Incorporated). IgM was always confined to fractions 2, 3 and 4 and these were pooled

to form the "19S" fraction. IgG and IgA were confined to fractions 6, 7, 8, and 9 in all tubes; these fractions were pooled to form the "7S" fraction. "19S" and "7S" fractions from each serum were dialyzed against PBS pH 7.5 for 24 hours, then brought to 1.5 ml total volume with PBS so that each immunoglobulin was at a 1:15 dilution compared to the original serum concentration.

SA absorption of unfractionated serum and 19S and 7S fractions: Aliquots of a 1:15 dilution of whole serum in PBS and the 1:15 dilutions of 19S and 7S protein were absorbed twice with staphylococci. Absorbed and unabsorbed aliquots were tested for blocking activity in the CBA.

2-ME treatment of unfractionated serum and 19S and 7S fractions: To 0.2 ml aliquots of 1:15 dilutions of serum, 19S antibody and 7S antibody, 14 microliters of a 10% dilution of 2-mercaptoethanol in distilled water (wt/wt) were added, to give a final concentration of 0.1 molar 2-ME. After mixing and incubating for 1 hour, the treated and untreated aliquots were assayed for CBA blocking activity. No attempt was made to dialyze away excess 2-ME; we assumed that the 1:20 dilution of the specimen in the first step of the HAVAB (R) assay would adequately dilute the 2-ME.

Diagrammatic summary of protocol: Figure 67 is a diagrammatic summary of the protocol.

Statistical analysis of data: P values were assigned to differences in sample means between untreated and treated serum and serum fraction groups using Student's t test for paired observations (176).

RESULTS: Results are presented in tabular form in Table 105 in graphic form in Figure 68.

In describing the staph absorption technique for detecting acute phase anti-HAV antibodies, Bradley et al (171) expressed their results as the ratio of (raw CPM absorbed/raw CPM unabsorbed). With unfractionated sera they found a ratio <2.0 only in acute sera and not in convalescent specimens. However, to achieve consistent results each serum specimen required variable and unique dilution before absorption.

We believe that this variable dilution step was unnecessary. By calculating the results as:

Table 104 Anti-HAV Antibody Content of Group N and Group A Sera

Serum #	pt. age	Sex	Day of illness serum obtained	HBsAg	% blocking undiluted	1:15 serum dilution: % blocking		Abs/unabs activity ratio
						Unabsorbed	Absorbed	
Group N	5	F	6	+	98%	74%	66%	.89
	6	M	43	-	96%	82%	70%	.86
	10	F	"Acute"	-	93%	73%	60%	.82
	3	M	2	-	90%	66%	62%	.94
	17	F	5	-	98%	79%	63%	.80
	10	F	2	-	90%	64%	50%	.78
	21	M	4	-	ND	73%	67%	.91
Group A	15	F	31	-	97%	75%	-03%	-04
	6	F	2	-	87%	63%	07%	.11
	18	M	21	-	99%	75%	32%	.43
	23	F	30	-	98%	73%	00%	.00
	27	M	3	-	ND	97%	07%	.10
	21	M	2	-	ND	72%	04%	.06
	21	M	11	+	98%	77%	08%	.11

Table 105 Percent Inhibition by Serum and Serum Fractions in
HAVAB (R) (% Inhibition = $\frac{\bar{X}NC - \text{Test}}{\bar{X}NC - \bar{X}PC}$)^a

	Serum #	Unfractionated Serum			7S Fraction			19S Fraction		
		UN	SA	ME	UN	SA	ME	UN	SA	ME
Group N	63064	61	52	48	39	36	34	58	41	32
	64037	80	71	65	66	40	54	36	42	18
	63960	73	57	43	44	31	37	50	37	21
	64024	64	57	41	31	21	26	46	38	14
	62959	68	58	45	25	12	28	28	24	24
	64036	79	68	58	53	37	40	48	39	12
Group A	64053	77	16	73	68	08	73	00	6	24
	63062	57	06	35	22	00	32	14	21	34
	63114	84	31	82	62	13	62	04	06	11
	63080	59	05	62	32	-11	39	15	08	20
	63162	35	06	43	28	-21	25	16	02	06
	63233	46	12	50	18	07	11	07	04	18
Controls	64004	52	-09	56	37	23	39	-04	00	05
	63247	-02	05	22	17	12	04	-02	03	21
	63285	12	29	10	16	03	16	08	10	23

a. $\bar{X}NC$ =mean negative control counts per minute

$\bar{X}PC$ =mean positive control counts per minute

Test=mean counts per minute in test using specified serum fraction

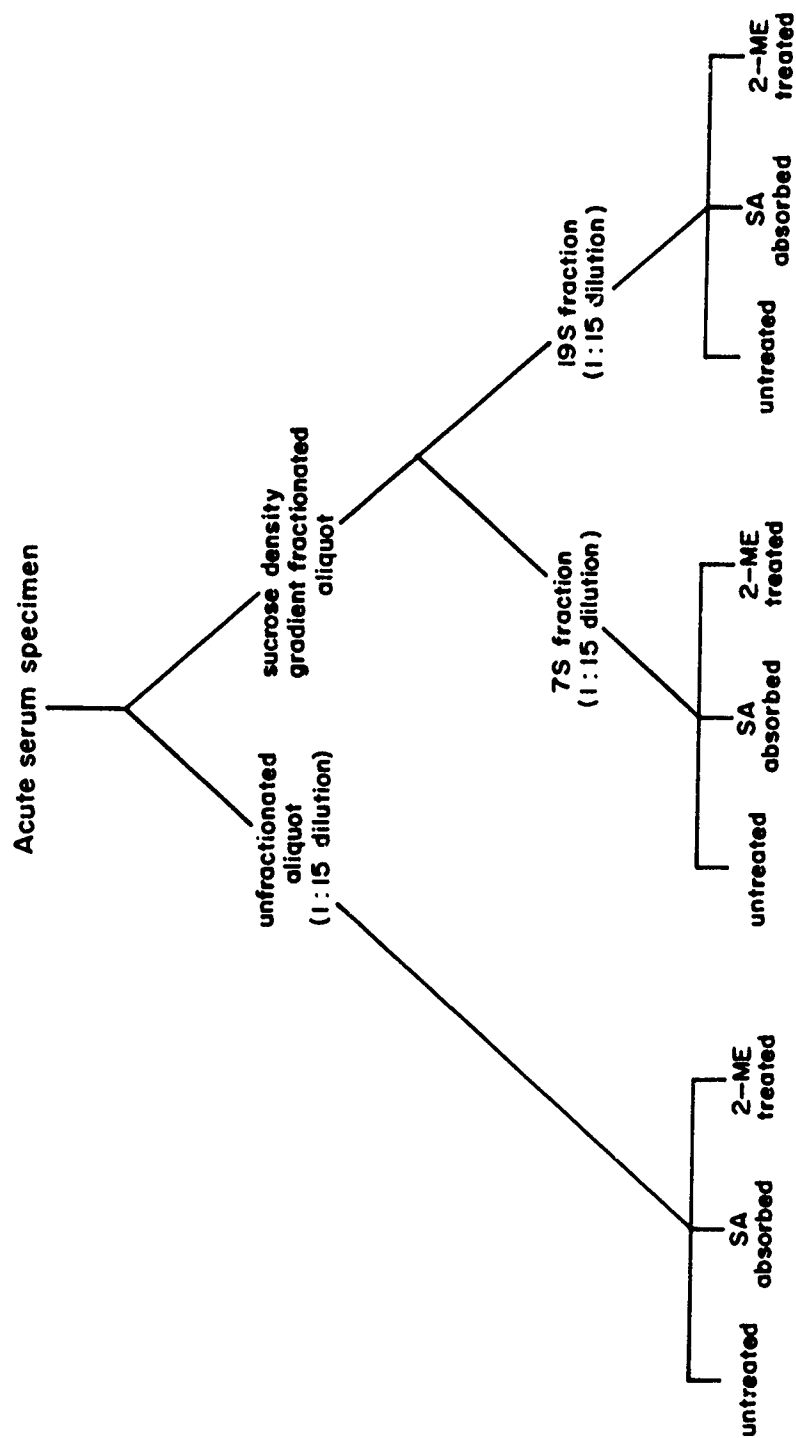


Figure 67. Diagrammatic summary of serum fractionation and treatment of serum fractions. SA absorbed=staphylococcus aureus absorbed; 2-ME treated = 2-mercaptoethanol treated. See text for details.

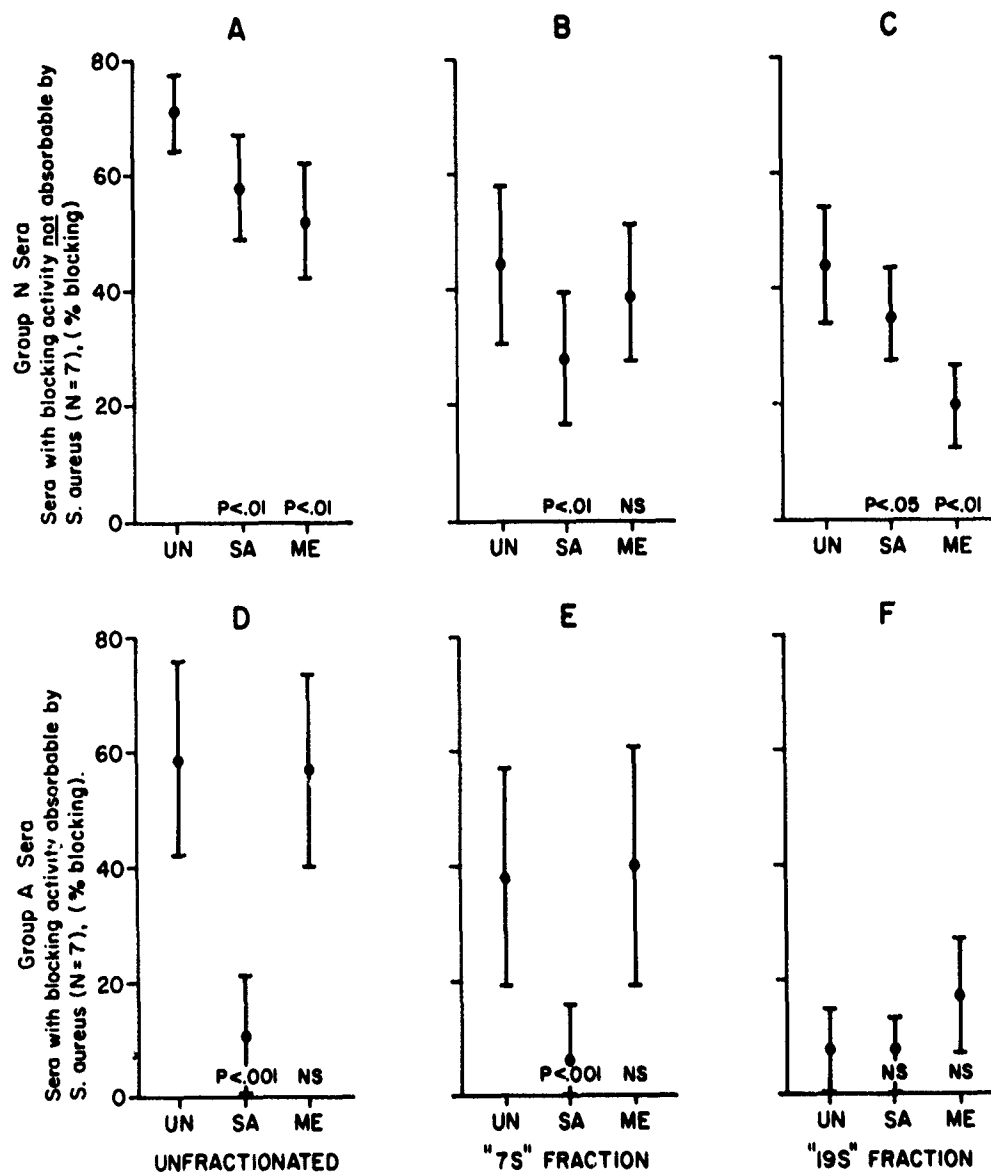
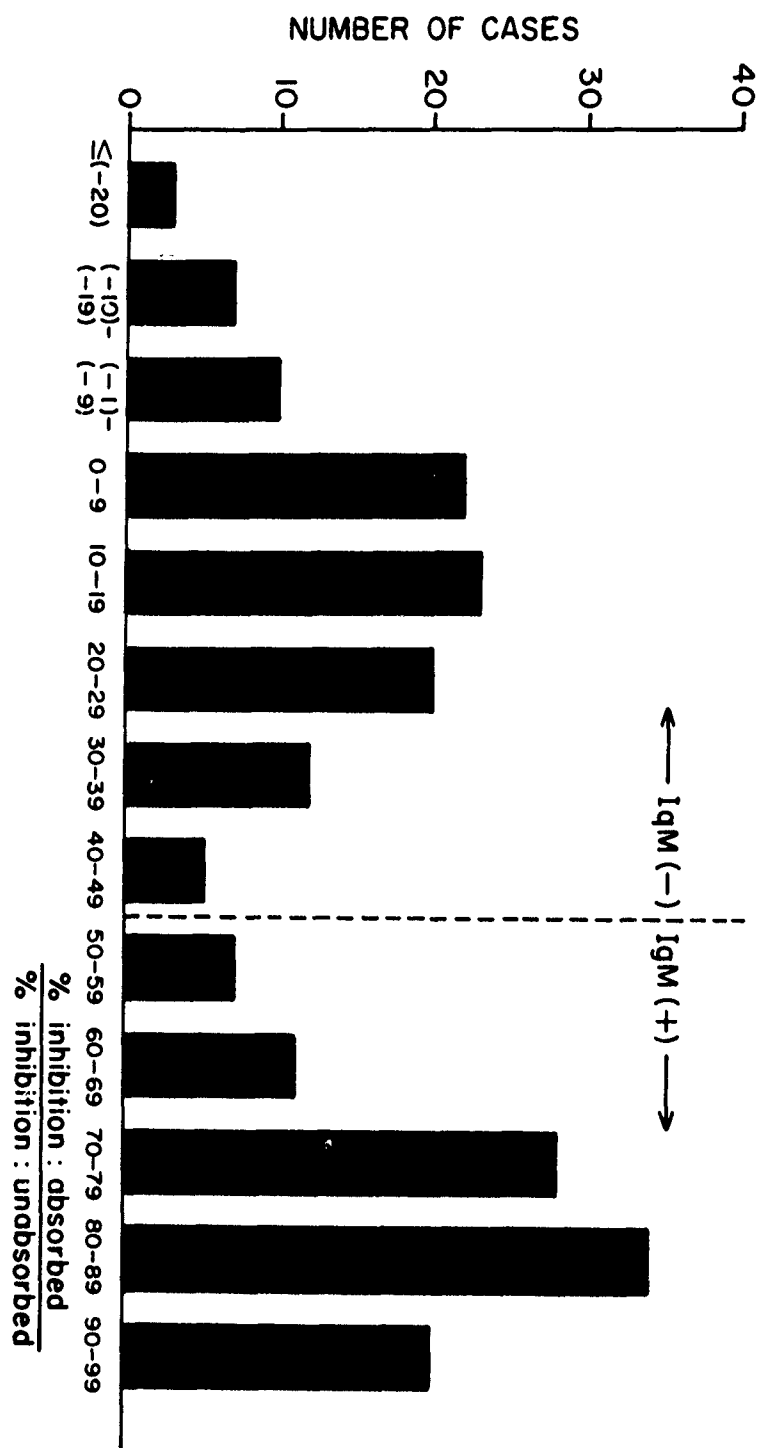


Figure 68 Graph of activity (% blocking) of unfractionated sera, 7S fractions, and 19S fractions from sera with SA absorbable activity (Group A) versus sera with activity not removed by SA absorption (Group N). A. Group N, unfractionated sera. B. Group N, 7S fraction. C. Group N, 19S fraction. D. Group A, unfractionated sera. E. Group A, 7S fraction. F. Group A, 19S fraction. UN = untreated; SA = absorbed with staphylococcus aureus; ME = treated with 2-mercaptoethanol. Mean \pm one standard deviation graphed. P values calculated for difference between treated specimens (either SA or ME treated) and untreated specimens.



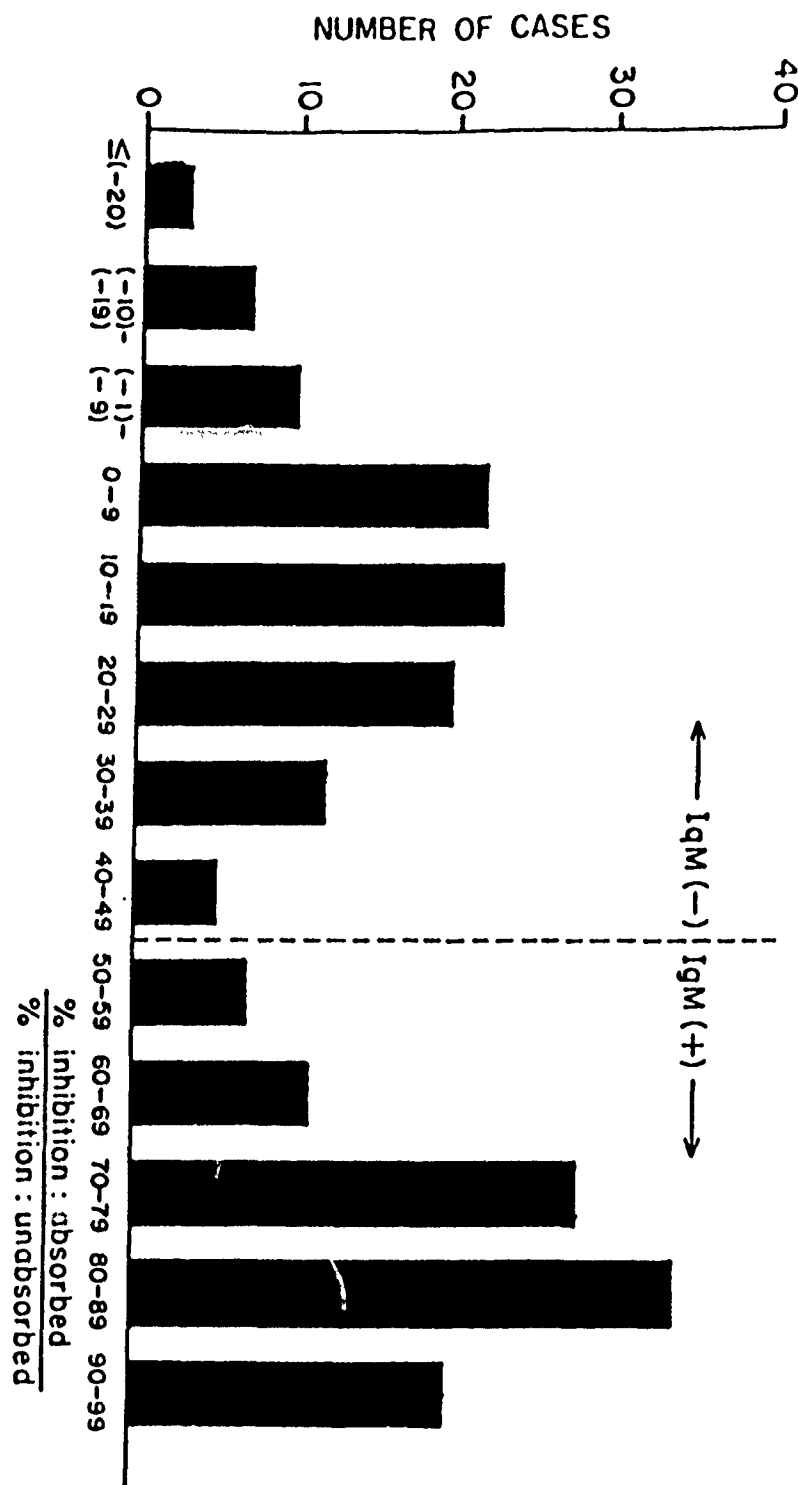


Figure 69. Histogram of distribution of acute sera according to the proportion of blocking activity remaining after SA absorption. Negative ratios denote sera in which the CPM in absorbed sera were greater than in negative control sera.

$$\frac{\% \text{ inhibition : absorbed}}{\% \text{ inhibition : unabsorbed}} = \frac{\frac{(\text{CPM:NC-CPM: test absorbed})}{(\text{CPM:NC-CPM: PC})}}{\frac{(\text{CPM:NC-CPM: test unabsorbed})}{(\text{CPM:NC-CPM: PC})}}$$

Where CPM = counts per minute; CPM:NC - CPM with negative control sera; CPM:PC - CPM with positive control sera.

$$\frac{(\% \text{ inhib absorbed})}{(\% \text{ inhib unabsorbed})} = \frac{\frac{(\text{CPMNC-CPM test absorbed})}{(\text{CPMNC-CPM PC})}}{\frac{(\text{CPMNC-CPM test unabsorbed})}{(\text{CPMNC-CPM PC})}}$$

We found that we could follow a set procedure, including a fixed pre-absorption dilution, and still clearly separate IgM positive and IgM negative sera.

Sera selected on the basis of non-absorbability of activity (Group N, inhibition ratio ≥ 0.5) fractionated into 7S and 19S fractions with $64 \pm 18\%$ and $62 \pm 18\%$ respectively, of the blocking activity of whole serum, while sera with absorbable activity (Group A, inhibition ratio < 0.5) fractionated into 7S and 19S fractions with $64 \pm 20\%$ and $17 \pm 17\%$ respectively. That the 19S activity in the Group N sera was due to IgM was proven by its sensitivity to treatment with 2-ME; 2-ME treatment had no effect on the 7S fractions.

In agreement with the results by Pastore et al (172) and Girardet et al (175), treatment of whole serum with 2-ME produced a decrease in activity of specimens in group N but not of those in group A. However, the magnitude of the decrease in the activity of group N sera produced by 2-ME treatment ($27 \pm 10\%$) was less than that produced by staph absorption of group A sera ($84 \pm 12\%$).

We initially thought that most of the activity in the group N sera not inactivated by 2-ME was probably IgG; however, considerable activity remained in the 7S fraction of the group N sera even after SA absorption, while SA absorption of the 7S fraction of group A sera almost completely removed activity ($36 \pm 18\%$ removed vs $81 \pm 24\%$ removed). As determined by immunodiffusion; both IgG and IgA were present in our 7S fractions; the non-absorbable 7S activity may be due to either IgA or to a high concentrations of non-absorbable IgG (?IgG₂) anti-HAV in acute sera from patients with HAV virus infections.

Using the staph A absorption method with a fixed dilution of serum before absorption, we have now examined samples of anti-HAV positive blood from over 200 patients with acute hepatitis. A histogram of the distribution of patients according to the ratio (% inhibition absorbed/% inhibition unabsorbed) is presented in Figure 69. The distribution is clearly bimodal adding further proof that the SA absorption modification of the CBA does crisply differentiate between sera with acute versus chronic anti-HAV activity.

40. Determination of the Etiology of Acute Hepatitis Infections in Bangkok: A Pilot Study

OBJECTIVES:

1. To estimate the relative incidence of clinically diagnosed acute hepatitis due to virus types A, B, and Non A-Non B.
2. To collect clinical specimens from cases of acute hepatitis to be used as sources of antigen and antibody in the development of diagnostics tests for hepatitis A and Non A-Non B.

BACKGROUND: In 1971, a year-long survey of the role of Hepatitis B virus in acute hepatitis in Bangkok was conducted (177). Over most age ranges, HBsAg was detected by CIE in approximately 40-50% of serum specimens from patients. As the CIE method used has been shown to detect HBsAg in 80-90% of cases of hepatitis due to Hepatitis B virus, it is reasonable to estimate that overall 50-60% of cases of acute hepatitis were due to the hepatitis B virus.

Recently we determined the age-specific prevalence of antibody to hepatitis A in a Thai population, using a sensitive commercially available radioimmunoassay; 98% of serum specimens in a random sampling of adults from the metropolitan Bangkok area were positive for antibody to hepatitis A.

Based on these data, it is likely that the vast majority of adults resident in Bangkok are immune to Hepatitis A by virtue of childhood infection.

The availability of a sensitive test for IgM antibodies to hepatitis A (staph aureus absorption modification of HAVAB) now allows us to define the relative incidence of acute viral hepatitis due to hepatitis viruses A, B, and (by exclusion) NonA-Non B. There is no reason to believe that the role of hepatitis B virus has significantly changed over the past 7 years; it is probable that 50-60% of cases are still due to hepatitis B. Further, as almost all adults are immune to hepatitis A virus, it is reasonable to predict that Hepatitis Non A-Non B may be an important cause of acute hepatitis among adults in Bangkok.

MATERIALS AND METHODS: Clinical: A target of 200 cases of acute hepatitis was set for this pilot study. Clinical cases were sought from throughout the greater Bangkok area during the period

1 November-31 May 1979. Letters requesting assistance in collecting clinical materials were sent to 12 Bangkok physicians known to have an interest in clinical management of hepatitis. All cases clinically diagnosed as acute viral hepatitis by these referring physicians were included in this study. Specific criteria (e.g. total bilirubin 2.0, etc.) were not set; rather the diagnosis rested entirely on the judgement of the referring physician. The following clinical specimens were collected from each patients:

<u>Day</u>	<u>Specimens</u>
Day 0:	Serum #1 and stool
Day 14-30:	Serum #2
Day 30-90	Serum #3

On the day that a specimen was to be collected the referring physician called the investigators at the Department of Virology, AFRIMS, who in turn dispatched a nurse's aid to collect the specimen on that day.

At the time of diagnosis (Day 0) and the time of the second convalescent blood specimen the referring physician was requested to fill out a short questionnaire.

Laboratory: All acute sera were tested for the following: (1) HBsAg by CIE and AUSRIA-II (R) (2) HBs Antibody by AUSAB (3) Hep-A-Antibody by HAVAB (R) (4) Anti-HAV IgM by the staph aureus absorption modification of the HAVAB.

As a minimum, all convalescent sera were tested for the following: (1) Anti-HBs by AUSAB (2) Heterophile antibodies by Monocheck (R) (Hyland).

RESULTS: Two hundred and forty-six patients with acute hepatitis were entered in the study. The age distribution both of the total population studied and by individual referring hospital is shown in Figure 70. The large peak at age 20-21 probably reflects the mean age of the military patient populations at two of the referring hospitals.

Laboratory studies are completed on serum specimens from 223 patients. The etiology of acute hepatitis by 5 years age groupings is presented in Table 106. The general pattern suggests that HAV is the etiologic agent of most acute hepatitis in children under

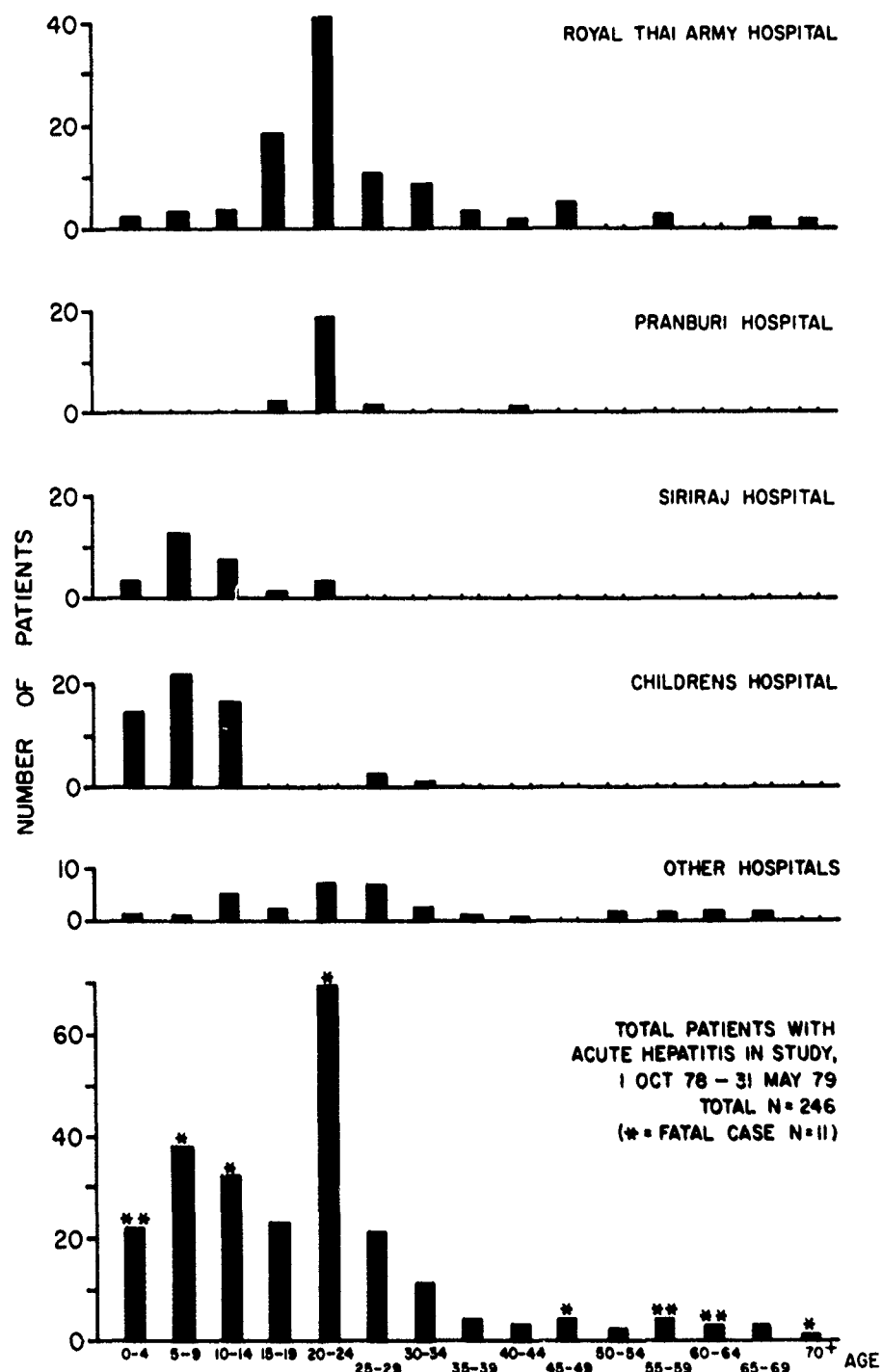
Table 106 AFRIMS Acute Hepatitis Study: 5 Year Age Groupings

Years of age	Total patients in age group	Evidence for active infection with:			
		HAV	HBV	HAV and HBV	NON A-NON B
0-4	18	11(61%)	4(22)	2(11)	1(06)
5-9	36	28(78)	0(0)	3(08)	5(14)
10-14	27	17(63)	5(19)	1(04)	4(15)
15-19	21	3(14)	7(33)	3(14)	8(38)
20-24	66	13(20)	41(62)	1(02)	11(17)
25-29	21	6(29)	9(43)	0(0)	6(29)
30 +	<u>34</u>	<u>4(12)</u>	<u>15(44)</u>	<u>0(0)</u>	<u>15(44)</u>
	223	82(37)	81(36)	10(05)	50(22)

Table 107 AFRIMS Acute Hepatitis Study: Mortality by Virus Type

Virus type	#Fatal/ Total	%Fatal	Age of fatal cases
HBV	4/81	5%	(57, 61, 21, 46)
HAV	0/82	0%	-
NANB	5/50	10%	(6, 78, 56, 11, 62)
HAV + HBV	0/10	0%	-
Not complete	2	-	(9/12, 2)

Figure 70 Source of Clinical Specimens in Acute Hepatitis Study



15 years old; that HBV is the most important cause in the young adult population; and that HBV and HANB are predominant in older adults.

Overall 11 of the 246 studied cases were fatal. Fatalities tended to occur at the extremes of age (4 death \leq 11 yo., 1 death 12-45 yo, 6 death \geq 46 yo). Fatality rates for each type of hepatitis are shown in Table 107.

A more detailed prospective study of all patients presenting to the Pramongkutklo Royal Thai Army Hospital with a diagnosis of acute hepatitis is currently underway.

41. Simultaneous Infection with Hepatitis A Virus (HAV) and Hepatitis B Virus (HBV)

OBJECTIVE: To identify and describe the laboratory findings in cases of acute hepatitis in which there is evidence for simultaneous infection with HAV and HBV.

BACKGROUND: Both HAV and HBV are endemic in Bangkok. Essentially 100% of adults show evidence of previous infection with HAV; 5-10% of native Thais of all age groups are carriers of HBV and an additional 50% of antigen-negative adults have anti-HBs.

On the basis of these facts, we reasoned that dual infections with both HAV and HBV are probably not unusual in Bangkok. As most research work on HAV has been conducted in developed countries where incidence rates of hepatitis are much lower than in Bangkok, human cases of simultaneous infection have been infrequently reported (178). In the laboratory, an experimental chimpanzee which was reported to have had an inadvertent infection with both viruses, developed an unusually severe illness (179). In the course of our preliminary acute hepatitis protocol, we tested serum specimens from patients for evidence of infection with HAV and/or HBV.

Overall results are presented elsewhere in this Annual Report. Ten cases were found with suggestive evidence of simultaneous HAV and HBV infection; reported here are five well documented cases.

METHODS: Acute sera from 223 patients with acute hepatitis were screened for evidence of anti-HAV IgM by the staph absorption modification of the HAVAB(R) solid phase competition radioimmunoassay (SA-CBAM). Acute and convalescent sera were screened for

HBsAg by CIEOP and SPRIA (AUSRIA-II (P)) and for anti-HBs by CIEOP and SPRIA (AUSAB (R)). Acute and convalescent serum specimens from patients that showed evidence of recent or current infection with both HAV and HBV were further analyzed by sucrose density gradient fractionation. IgM positive fractions for each specimen were pooled and dialyzed against PBS as were IgG positive fractions. After centrifugation and dialysis, immunoglobulins were at a 1:4 dilution of their original serum concentration. Each fraction pool was then run in the HAVAB (R) SPRIA with or without 2-mercaptoethanol (2-ME) at a final concentration of 0.1M. To be included in this series each case required, as a minimum, the following laboratory proof of diagnosis:

<u>DIAGNOSIS</u>	<u>REQUIREMENT</u>
Acute HAV	<ol style="list-style-type: none"> 1. SA-CBAM assay positive on acute serum and 2. 19S fraction of acute serum shows 2-ME labile activity in HAVAB
HBV	<ol style="list-style-type: none"> 1. HBsAg (AUSRIA-II) positive in acute serum and 2. Anti-HBs (AUSAB) or HBsAg (AUSRIA-II) positive in convalescent serum.

RESULTS: Laboratory tests done to establish evidence of concurrent infection with HAV and HBV are presented above. Of the five cases, three appear to have occurred in chronic HBs Ag carriers, while in two cases acute infection occurred simultaneously with both viruses. The clinical histories as recorded on the protocol questionnaire forms of these five patients were not extraordinary. None of the patients died, and none had evidence of pre-coma. Further details have been requested from the attending physicians.

42. Epidemiologic Investigation of Cases of Acute Hepatitis Among Troops of the Thanarat Army Base, 30 Oct-2 Nov 78

OBJECTIVE: To conduct epidemiological studies of the etiology and mode of transmission of cases of acute hepatitis at the Thanarat Army Base, and, based on the findings, to make recommendations for hepatitis control.

BACKGROUND: On 3rd October 1978, COL Wittoon, Dr. Rapin and MAJ Burke met at AFRIMS to discuss collaborative laboratory and epidemiologic investigations aimed at curtailing an apparent epidemic of acute hepatitis at the Thanarat Army Base. After obtaining authorization for collaborative investigations, COL Wittoon invited a field research team to the Thanarat Army Base for the period 30 Oct-2 Nov 1978.

The Thanarat Army Base is located in the town of Pranburi in the Province of Prachuap Khiri Khan, about 250 kilometers south of Bangkok on the Gulf of Thailand at the mouth of the Pranburi River. The civilian population of Amphur Pranburi is approximately 90,000. Income is derived from agriculture (growing pineapple, sugar cane, and coconuts) and from fishing.

Four battalion-sized units are billeted at the Thanarat Army Base. Three are infantry units, while one is a special school not within the Infantry Corps command structure (Figure 71). Although data in unit strength was made available to the epidemiologic team for purposes of analysis this information will not be reported here for reasons of security. The four units are:

I. Infantry Combat Battalion. This unit is comprised of men who have completed infantry training. This battalion is mobile and has spent most of the past two years in combat with insurgents in the southern provinces of Thailand.

II. Infantry NCO School. Until recently, this battalion-sized unit had the function of providing a one-year course of specialized infantry training for men who had already completed one year of general NCO training (2nd year students). However, due to a shortage of infantry NCO's, this year a special intensive course was implemented to train men in infantry skills who have had no previous NCO training (1st year students). A support company is attached to this unit.

III. Infantry Center Support Battalion. This unit is comprised primarily of privates who provide non-skilled support services for the Infantry Center (guards, motor pool, headquarters, staff, etc.).

IV. Permanent NCO School. This unit has no specialized infantry function, and does not fall under the direct Infantry Corps command structure. The permanent NCO school is a base tenant organization that teaches cadets (80% directly from civilian life, 20% from the ranks of enlisted men throughout Thailand) Basic NCO skills (math, language, basic firearms, etc.). The course lasts for one year, and graduates usually go to a special corps-specific NCO school for a second year of training. This unit has a support company.

These four major units turn over personnel at different rates and at different times of the year. Personnel in the Infantry Combat Battalion are rotated only sporadically; the second year Infantry NCO class changes 100% every May; the intensive first year course of the Infantry NCO school began with its first class in August 1978; the permanent NCO school turns over 100% each May; and the support companies and the Support Battalion, turn over approximately 25% of their personnel every May and November.

These personnel rotations are diagrammed in Figure 72.

Medical care facilities available at the Thanarat Army Base included the main hospital, with its two small satellite dispensaries, and an administratively separate dispensary at the permanent NCO School. Manpower and patient beds at each of the facilities are summarized in Table 108. Although soldiers at the base typically seek health care first at their own unit's dispensary, all facilities are open to all soldiers, so that Infantry School Students are occasionally hospitalized at the permanent NCO school dispensary, and permanent NCO school students are also occasionally admitted to the main hospital. The more seriously ill patients admitted to the permanent school dispensary are often transferred to the main hospital for more sophisticated treatment.

As the nearest provincial hospital is 30 kilometers from Pranburi, the military hospital serves as a major provider of health care of the civilian community as well as the military population. Nonetheless, the Pranburi military hospital's primary mission is to provide comprehensive health care for the active duty soldiers at the Base.

METHODS: Team description: The AFRIMS investigative team consisted of three physicians (two virologists and one public health specialist) and two field technicians. On-site support was provided by the medical staffs of the Infantry Center and the Infantry School. Laboratory support was provided by the Department of Virology, AFRIMS.

Personnel Interviewed: The following installation personnel contributed materially to the investigation:

- Post Commanding Officer
- Post Deputy Commanding Officer
- Assistant Chief of Staff-Intelligence
- Post Surgeon

Chief, Facilities Engineers
Infantry School Surgeon
Post Environmental Health Officer

Case Ascertainment: From the hospital and dispensary census registries a list was prepared of all patients admitted with a diagnosis of "viral hepatitis" (57 patients) or "jaundice" (5 patients). Currently hospitalized patients were examined. In addition, the records of 24 of these 62 patients were examined, and, with one exception, were all compatible with viral hepatitis. The single exception was the sole fatality, the autopsy diagnosis being "acute leukemia." This case will not be considered further in this report. Cases of "jaundice" were not hospitalized routinely. Mild cases, especially in officers, senior NCOs, and dependents, would probably not be hospitalized. Outpatient records were inadequate to ascertain the number of cases treated in this manner.

Control Selection: From the inpatient registries, controls were selected for each hepatitis patient. The control was selected as the next patient listed in the admission log book after a hepatitis patient. Dispensary controls were sought for dispensary admissions and hospital controls for hospital admissions. Where the nearest admission control was unavailable (TDY, transfer from post, etc.) the next closest admission was sought. Questionnaires and serum samples for these individuals were treated in the same manner as those of hepatitis cases.

Case and Control Investigations: Each case and control located had a serum specimen drawn for serologic studies. In addition, each case and control filled out a questionnaire (Appendix A). The questionnaire identified the study participant as to military unit of assignment, age, and rank. Questions were also asked regarding previous residences, occupations, exposure to hepatitis patients, and activities which may have placed the individual at unusual risk of infection with hepatitis virus.

Laboratory Procedures: Serum specimens from cases and controls were tested as follows:

Anti-hepatitis A antibody: Sera were screened undiluted with a competitive solid phase radioimmunoassay (HAVAB, Abbott Laboratories).

Anti-HAV IgM: Sera were diluted 1:20 with PBS, absorbed twice with whole staph aureus and assayed for residual activity in the HAVAB assay.

Anti-HBs antibody: Sera were screened undiluted by solid phase radioimmunoassay (AUSAB, Abbott Laboratories) and undiluted by counterimmunoelectrophoresis (CIE).
Hepatitis B surface antigen: Sera were tested undiluted by AUSRIA-II (Abbott Laboratories) and CIE.
Specimens positive for antigen by CIE were typed by immunodiffusion against specific rabbit antisera.
Anti-leptospirosis antibody: Sera were screened in an immune hemolysis test with 12 serotypes of leptospira (L. bataviae, icterohaemorrhagiae, hebdomadis, javanica, grippotyphosa, canicola, autumnalis, pomona, pyrogenes, australis, hyos and wolssi). Leptospirosis serology was performed in the laboratory of Dr. Sansiri Sornmanee at the Faculty of Tropical Medicine, Mahidol University.

Review of referrals for drug abuse: Records of all referrals of active duty personnel to the post surgeon for drug abuse treatment and counselling during the preceding year were reviewed, with special attention to the type of drug abuse and unit assignment of the referred soldiers.

Red Cross Blood Donations: Results of Red Cross CIE HBsAg screening of blood donors, as recorded on individual donor cards at the Thanarat Army Base during the preceding year, were reviewed, and the proportion of donors positive tabulated for each company or battalion during each donation period.

Rosters of the order in which NCO permanent school students received immunizations on entrance into the school in May 1978 were obtained. Records of the August 1978 permanent NCO school blood donations were reviewed, the position on the immunization sequence roster of each HBsAg positive donor was noted, and an analysis for "clustering" of cases HBsAg positive donors was conducted.

RESULTS: Distribution of Cases Over Time: Figure 73 presents the hospital (or dispensary) admissions for "viral hepatitis" or "jaundice" between November 1977 and October 1978. November 1977 through April 1978 showed a "baseline" level of 1-4 cases per month. May had a slight increase in cases, especially in the latter half of the month, and July-September 1978 had the highest three month total of cases seen on this post within the observation period.

In an effort to determine the variance of this seasonal distribution from normal, the distribution of cases for the previous 12 month period (Nov 76-Oct 77) was ascertained. Records prior to

October 1976 were unavailable due to a change in medical facilities at that time.

As Figure 74 shows, a similar seasonal distribution was noted in 76-77, with relatively low levels during the cool and hot, dry seasons (Nov-Apr) with increased cases during the rainy season (May-Oct) and a year-high peak in the late rainy season. The 76-77 period did not have the magnitude of cases exhibited in 77-78, but the seasonal distribution was similar.

Distribution of Cases by Unit: For the purposes of this report, the post can be divided into six major units. There were some small units on post, but the sum of men assigned to them was less than 100 and they sustained no hepatitis hospitalizations, nor were controls selected from them.

During the course of the year November 1977-October 1978, the hepatitis attack rates of five of the six units were approximately equal, ranging from 6.0 to 9.5 cases per 1000 men. The support troops for the Infantry School, however, sustained a rate of approximately 17 cases per 1000 men. The cases in this unit were moderately localized by the time, there being 1 case in December 1977, 1 in January, 2 in May, 1 in June, 2 in July, 3 in August, and 1 in September. While this unit represented slightly less than 10% of the post population, from December 1977 through January 1978, this unit contributed 25% of hepatitis cases and between May and August 1978, 33% of the cases occurring on post.

Admission for hepatitis by month for each major unit are graphed in Figure 75.

Clinical Description of cases: Clinical hospital and laboratory charts of 24 patients were reviewed. Although not detailed, the charts contained sufficient information to reconstruct the following "typical patient."

Symptoms: Malaise, nausea, vomiting, abdominal distension, headache, dark urine

Signs: Fever, jaundice, hepatomegaly and tenderness of hypochondriac region

Records of certain other important symptoms and signs with significance for establishing a differential diagnosis were sought, but not found.

Specifically lacking was mention of significant meningismus, sore throat, lymphadenopathy, rashes or conjunctivitis. Available liver function test data are summarized in Table 109. Of the 24 patients whose records were available for review, the mean SGOT was 101.9 SFU and mean total bilirubin was 6.7 mg%.

There were no deaths among the 62 cases of hepatitis. One case was diagnosed as progressing to hepatic pre-coma. Although exact data is lacking, the average soldier was discharged from the hospital or clinic within 9.5 days after the onset of clinical illness.

Serology: Results of 37 patients and 36 paired controls are summarized in Table 110. Hepatitis B surface antigen was detected in 11% of patients and 9% of controls; two patient's and one control sera contained sufficient HBsAg for subtyping; all three were subtype adr. Anti-hepatitis B antibody was detected in 25% of patients and 42% of controls. Anti-leptospirosis antibody was not detected in any member of either the patient or the control populations. Anti-hepatitis A virus antibody (anti-HAV) was detected in 100% of patients and 42% of controls. Of 34 case control pairs in which sufficient serum was available to test for anti-HAV IgM by the staph absorption modification of the HAVAB (R), anti-HAV IgM was found in serum from 10 cases but only 1 control ($\chi^2 = 8.78$, $p = .003$). Of 17 case sera from patients who had been ill during the period July through September 1978, 7 were positive for anti-HAV IgM, while only 3 of 19 drawn from patients who had been ill before July were positive.

Paired blood specimens were available from three hepatitis patients. One (pt#39) contained HBsAg in both acute and convalescent sera with no anti-HAV titer rise, while two patients (#56 and #76) were negative for HBsAg and anti-HBs, and showed non-diagnostic (0.5 log) rises between acute and convalescent anti-Hav titers.

Red Cross HBsAg screening of blood donors: Results of Red Cross HBsAg screening of active duty blood donors at the Thanarat Army Base during the preceding year are summarized in Table 111. Unit prevalence ranged from 0.029 to 0.121. Prevalence among the permanent NCO school students varied considerably through the year; rates in October 1977 and August 1978 (0.087 and 0.089) were significantly higher than in December 1977 (0.029), $p < 0.05$ by chi-square test with Yates correction.

Of the 91 HBsAg positive permanent NCO school donors in August 1978, 79 were located on the May 1978 immunization sequence rosters. On the assumption that the 79 HBsAg-positive blood donors were randomly distributed among the total of 1714 personnel, a normal distribution of the "distance"* between individuals was set up with $X = 21.4$ and $u = 18.24$. Table 112 presents the expected and observed frequency of the various "distances." The distribution of observed frequencies does not fit a normal distribution (Goodness of fit $\chi^2 = 11.03, df = 1, p < 0.005$) because of the excess cases in the 5-7 person class. As immunization practices usually involve 10 persons/needle change, the above excess is compatible with (but not necessarily demonstrative of) person-to-person spread of HBsAg positive blood through contaminated needles.

Drug Abuse Referrals: Eighteen active duty troops were referred to the Post Surgeon during the preceding year for treatment and counseling of drug abuse. All cases involved illicit intravenous injection of heroin. Table 113 summarizes the rate of referrals from each of the six main units on post. Rates ranged from referrals among the permanent NCO school students to 11 referrals per 1000 men per year among the personnel of the support company of the Infantry School.

Questionnaire Results: A total of 37 hepatitis patients were found and 32 matched case control pairs were identified.

Of the 32 pairs, the controls were slightly older (avg. 24.4 years vs 22.3 years) and had been stationed at Pranburi for slightly longer (avg. 2.94 years vs 2.02 years). The controls had been in the Army significantly longer than the hepatitis cases (avg. 4.75 years vs 2.02 years). Only two of the cases had been on active duty for more than two years compared to seven of the identified controls. In all cases, the hepatitis patients had spent their total time in service at this one post. The distribution of home provinces between the two groups was similar.

Between cases and controls no differences were found in total hospitalizations, medications taken, incidence of blood transfusion, medical injections, dental work, tattooing or promiscuous razor use. No statistical difference was noted in the frequency with which cases and controls had contact with another person with

*Distance, for the purposes of this investigation, is defined as the number of personnel between two HBsAg-positive individuals.

jaundice, but cases consistently had slightly higher rates of unit barracks and family contacts with other jaundiced individuals.

In two areas a difference between cases and controls was evident. Over 50% (17/32) of patients admitted extramarital sexual relationships. This was more than twice the rate of the control group (8/32). Controls donated blood more frequently (13/32) than cases (7/32).

Interviews and Observations: Interview with Chief Facilities Engineer: The Pranburi installation was designed and built approximately 20 years ago. Since then the post population has increase three-fold without a change in either the water supply or sewage treatment facilities except as noted below.

Water for all uses is derived from the Pranburi River which abutts the post to the south. The water treatment systems was designed for immediate chlorination and subsequent holding in a settling tank for at least 48 hours. Current demand is such that the water tank is bypassed and water flows directly from the treatment plant into the water mains. Chlorine is added based on the estimated flow, which is intermittent; derived as it is, from irregular user demand. Examinations of the water either for chlorine residual or BOD level are not performed.

A sewage treatment plant was built when the camp was originated, but due to local civilian demand, a trickling sand filter method was adopted soon after the camp opened. The single acre of land set aside for the filter is both inadequate to safely decontaminate the sewage from the post population and too close to the river which provides water for much of the local civilian community. The camp does draw its water upstream from the slow filter; however, the degree of tidal mixing is unknown.

Since the camp has opened, much of the unused land upstream from the camp has been planted in pineapple and sugar cane, and residences have been established directly upstream from the camp.

Interview with immunization clinic personnel: Immunizations are administered at the infantry school dispensary according to the following schedule:

	Cholera	Tetanus	Smallpox	Bloodtyping
On reporting to base	X	X	X	X
30 days		X		
60 days		X		
180 days	X			
365			X	

In May and November of each year, the soldiers are immunized by company, usually 500-750 men per day for two or three days. Usual practices for each injection follow:

Tetanus: The vaccine is drawn up in a 20 ml syringe. After the needle punctures the skin of first vaccinees, the syringe barrel pulled back, and the syringe contents examined for blood reflux. Two ml of vaccine are injected. If no blood was seen to reflux into the syringe, the needle is changed, and the procedure repeated for the next man until ten men have been vaccinated per syringe. If at anytime blood is seen to reflux into the syringe, the remaining vaccine is discarded and the syringe resterilized. (Disposable syringes are not available). Cholera: The procedures used are identical to those used for tetanus. Smallpox: The vaccine is administered with several scratches of a small scarification knife. Between vaccinations the blade is wiped clean with alcohol, and then the procedure is repeated. A blade is used until it becomes dull, usually after five to ten men. Bloodtyping: A lancet is used to puncture the finger of each soldier. Usually there are enough lancets for each soldier; on some occasions, when lancets are in short supply, they are cleaned with alcohol swabs and reused. The usual stock of needles is three to four dozen. Standard procedure on immunization days is to collect needles as soon as they have been used for one man and boil them for 10-15 minutes.

At the permanent NCO school dispensary, only cholera vaccination and blood typing are performed, and the clinic is active only in May (as a result of the annual rotation of personnel). Procedures used are virtually identical to those described above.

CONCLUSIONS:

1. At the Thanarat Army Base incidence of hospitalizations for a diagnosis of acute hepatitis probably peaks each year during the months of August through October.

2. A peak incidence of cases of 16 during the month of September 1978, was sufficiently above the baseline rate to be recognized.

3. Although excess cases were not confined to one battalion or company, the attack rate was highest among the support troops of the Infantry School.

4. The excess of cases during the 1978 rainy season was probably caused in large part by the hepatitis A virus. Some smaller proportion of cases was probably caused by the hepatitis B virus. Leptospirosis can be excluded with reasonable certainty.

5. The high ($\geq 10\%$) carrier rates for hepatitis B surface antigen observed in some units during blood donations cannot be clearly related to the excess of hepatitis cases as most patients did not show evidence of previous hepatitis B infection.

6. At present several mechanisms known to be associated with the spread of viral hepatitis exist at the Thanarat Army Base, including an outdated water supply system, illicit drug abuse, sub-optimal immunization practices, and transfusion of blood without screening for hepatitis B virus surface antigen.

7. There is a statistical clustering of hepatitis B surface antigen positive blood donors according to the sequence in which soldiers were immunized. This suggests but does not prove a casual relationship immunization practices and infection with HBV.

8. Actions which are likely to decrease the incidence of acute hepatitis and decrease infections with the hepatitis viruses in order of priority, are as follows:

A. Purchase extra syringes and needles so that mass immunizations can be carried out with meticulously sterilized equipment.

B. Complete scheduled replacement of water supply system.

C. Conduct an education campaign of medical personnel on the potential hazards associated with immunizations,

D. Monitor and carefully maintain sterilization equipment to insure that boiling temperatures are achieved and maintained.

E. Establish a program for screening all units of blood to be transfused for the presence of hepatitis B virus.

Table 108 Medical care facilities and personnel at the Thanarat
Army Base

	Physicians	Nurses	Nurses aides	Officers	NCO's	Inpatient beds
Main hospital	7	15	25	40	70	200
Infantry Combat Battalion dispensary	0*	0	0	1	6	0
Infantry School Dispensary	0*	0	0	1	6	0
.....						
Permanent NCO School Dispensary	1	3	0	2	10	50

* Physicians assigned to main hospital provide health care at
these dispensaries on a rotating basis.

Table 109 Summary of liver function tests of hepatitis patients

Total Bilirubin	Number of patients	SGOT	Number of patients
Less than 2 mg%	2	Less than 35 SFU	5
2 - 5 mg%	9	35-100 SFU	10
5 - 15 mg%	11	100-200 SFU	4
More than 15 mg%	1	200-500 SFU	5
Total	23	Total	24
Total Bilirubin \bar{X} =	6.7 mg%	SGOT \bar{x} =	102 SFU

Table 110 Results of serology tests for hepatitis A and hepatitis B

Pranburi

Patients Study No.	Acc.No.	HBsAg		Anti-HBs		Subtype	HAVAB
		IEOP	RIA	IEOP	AUSAB		
001	62981	-	-	-	-		+
006	62986	-	-	-	-		+
008	62988	-	-	-	+		+
009	62989	-	-	-	-		+
012	62992	-	-	-	-		+
013	62993	-	-	-	+		+
015	62995	-	-	-	+		+
016	62996	-	-	-	-		+
017	62997	-	+	-	-		+
018	62998	+	+	-	-	adr+eAg	+
019	62999	-	-	-	-		+
020	63000	-	-	-	+		+
021	63001	-	-	-	+		+
022	63002	-	-	-	-		+
025	63005	-	-	-	-		+
026	63006	+	+	-	-	weak Ag + anti e	+
027	63007	-	-	-	-		+
028	63008	-	-	-	-		+
029	63009	-	-	-	+		+
030	63010	-	-	-	+		+
031	63011	-	-	-	-		+
032	63012	-	-	-	-		+
033	63013	-	-	-	-		+
034	63014	-	-	-	-		+
035	63015	-	-	-	-		+
036	63016	-	-	-	+		+
038	63018	-	-	-	-		+
039	63019	+	+	-	-	adr, eAg	+
040	63020	-	-	-	+		+
041	63021	-	-	-	-		+
045	63025	-	-	-	-		+
047	63027	-	-	+	+		+
048	63028	-	-	-	-		+
050	63030	-	-	-	-		+
076	63082	-	-	-	-		+
077	63132	-	-	-	-		ND
056	63036	-	-	-	-		ND

Table 111 Summary of Red Cross HBsAg Screening of Donor Blood of Thanarat Army Base During the Past Year (Oct 77 to Present) Numbers in the Table Represent the Proportion of Donors Positive; Numbers in Parentheses Give Actual Data (#Positive/#Tested).

	Oct 77	Dec 77	Jan 78	Apr 78	Aug 78
Permanent NCO School Students	.087(44/502)	.029(21/701)	-	-	.089(91/1025)
Permanent NCO Support Company	-	-	-	-	.114(10/880)
Infantry School Students, 1st year	-	-	-	-	-
Infantry School Students, 2nd year	-	-	-	-	-
Infantry School Support Company	-	-	.051(5/88)	-	-
Infantry Center Support Battalion	-	-	.045(6/134)	.042(7/168)	-
Infantry Combat Battalion	-	-	.121(18/148)	-	-

* Used exclusively as donors to hospital blood bank; blood not tested for HBsAg.

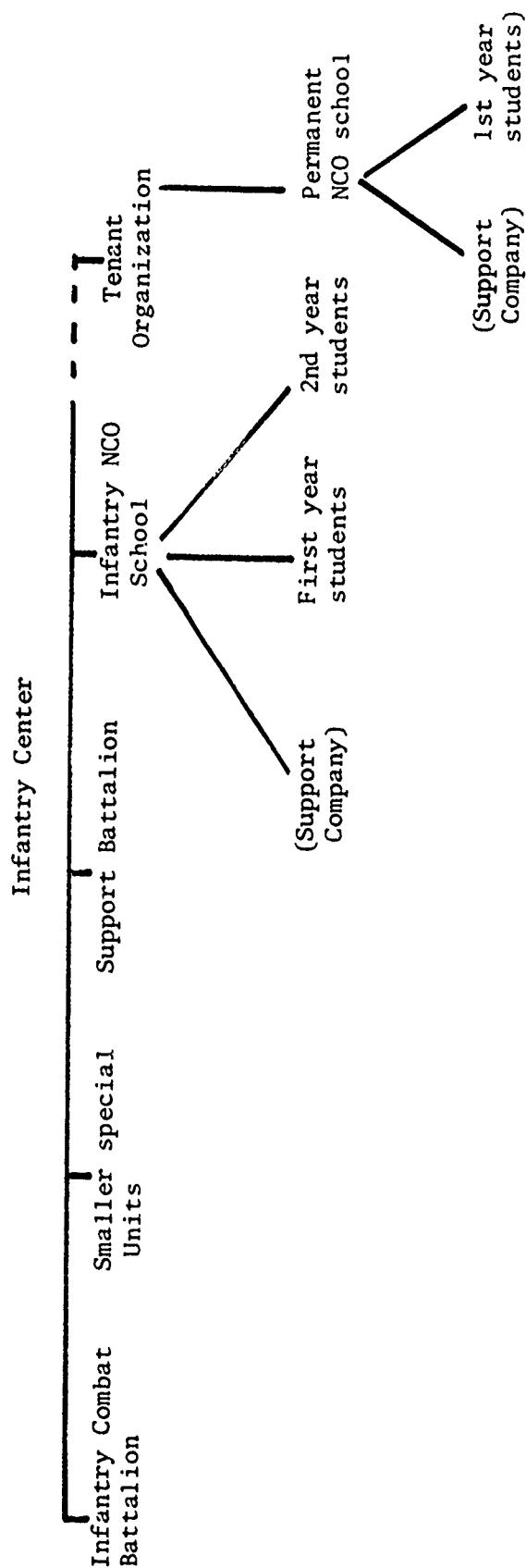
Table 1.12 Frequency Distribution of 80 "Distances" Between
HBsAg Positive Individuals. Pranburi Thailand,
August 1978.

<u>Class Limits</u> <u>(persons)</u>	<u>Observed</u> <u>Frequency</u>	<u>Theoretical</u> <u>Frequency</u>
≤ 4	12	14.7
5-7	7	2.5
8-9	6	2.6
≥ 10	55	60.2

Table 113 Drug Abuse Referrals

Unit Designation	Rate (referrals/man/year)
NCO Permanent School	.0000
NCO Permanent School Support Company	.0014
Infantry School	.0033
Infantry School Support Company	.0108
Infantry Center Support Battalion	.0030
Infantry Combat Battalion	.0031

Figure 71 Command structure diagram of units at the Thanarat Army Base

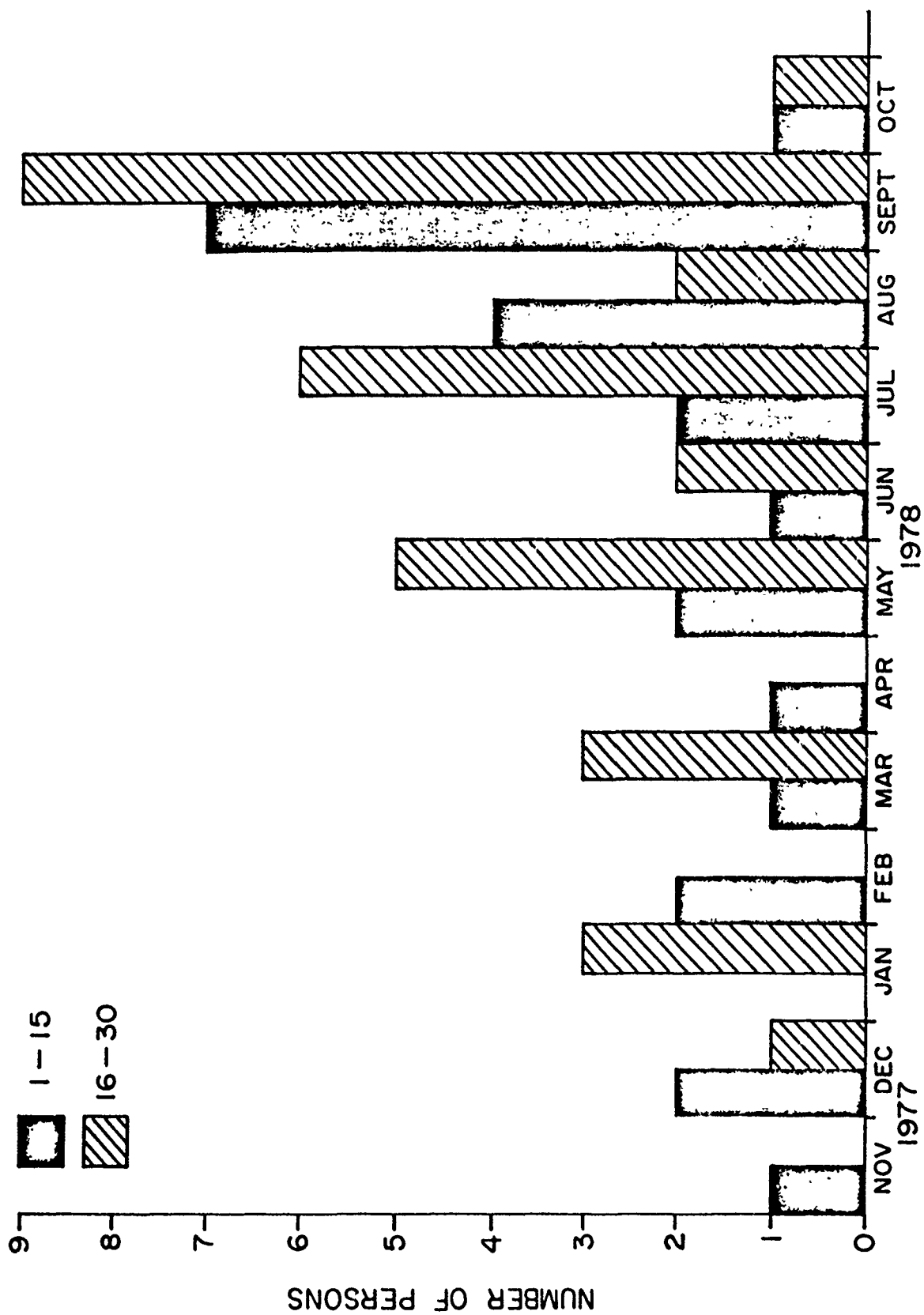


Health care provided by Infantry Center Medical Facilities (COL Wittoon Charoprakorn)

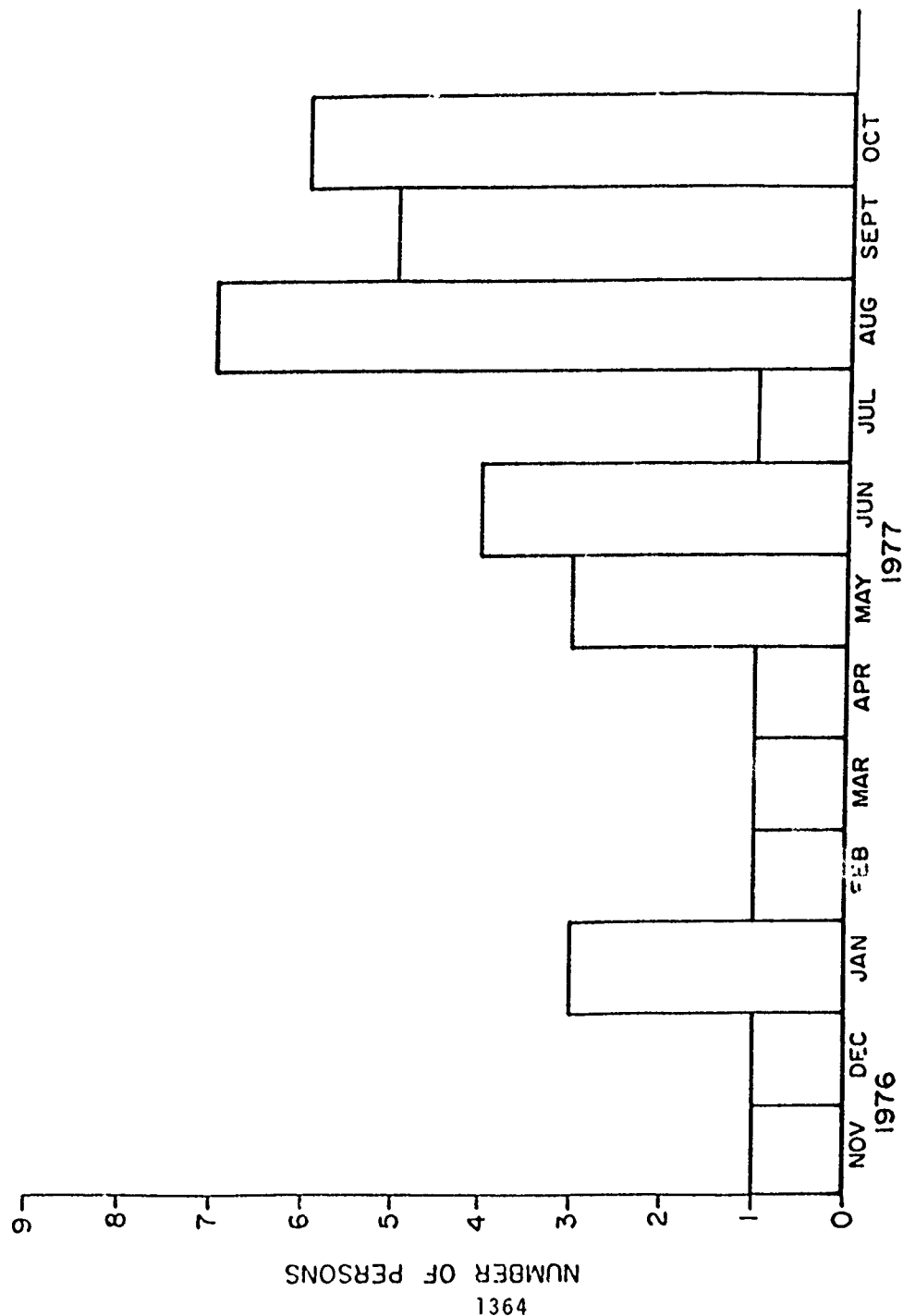
Health care provided by Permanent NCO school Medical Facilities (LTC Ittipol Senasen)

Figure 72 Rotation of Personnel at the Thanarat Army Base

	1977	1978
Infantry Combat Battalion		
Infantry NCO School 2nd year	May 100%	May 100%
Infantry NCO School 1st year	-----No Class-----	August
Permanent NCO School	May 100%	May 100%
Support Companies and Support Battalion	May 25%	Nov 25%
		May 25%



TOTAL ADMISSIONS FOR HEPATITIS BY HALF-MONTH, PRANBURI THAILAND.
Figure 73



TOTAL ADMISSIONS FOR HEPATITIS BY MONTH, PRANBURI THAILAND.

Figure 74

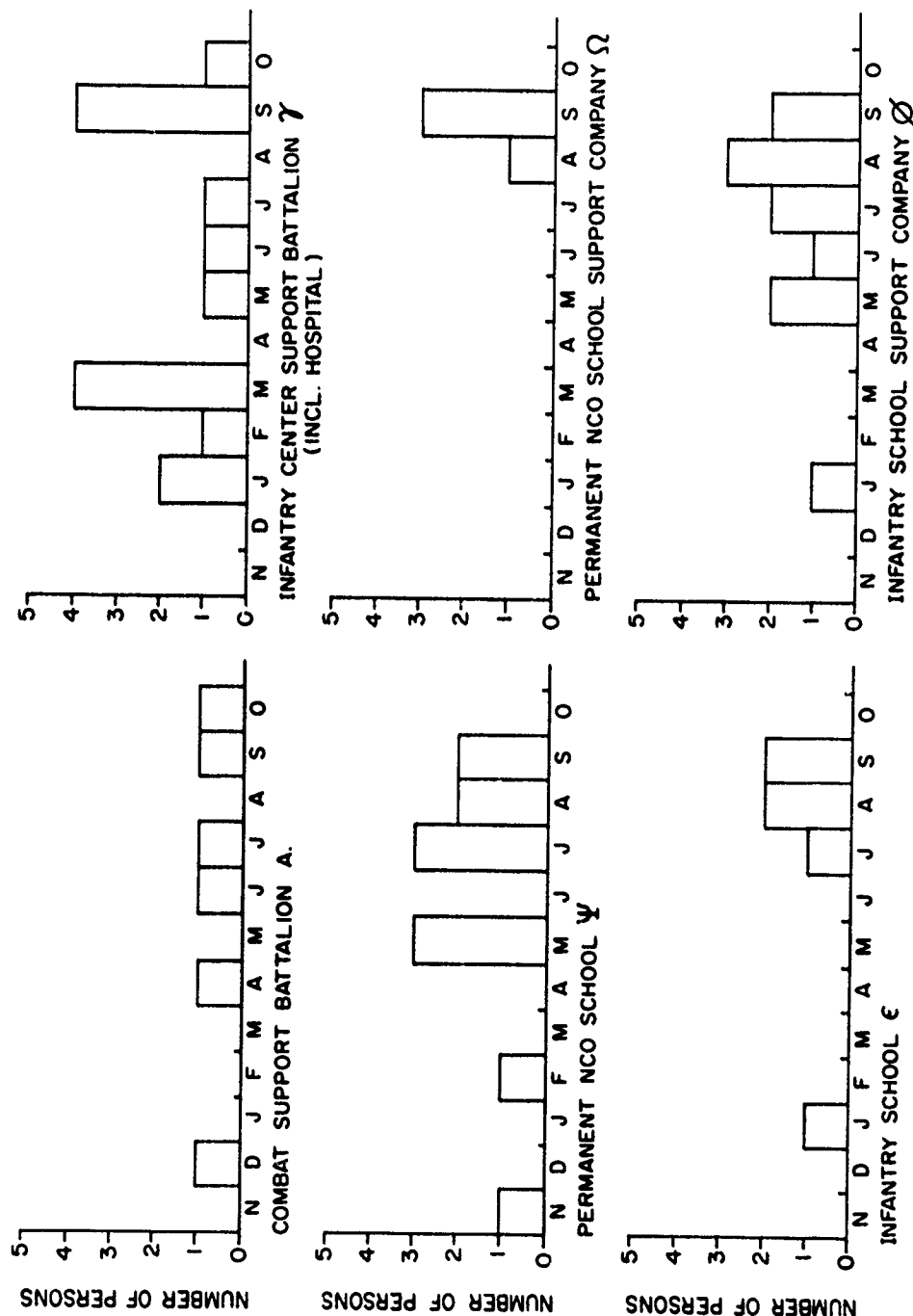


FIGURE 75 ADMISSIONS FOR HEPATITIS BY MONTH FOR EACH MAJOR UNIT, PRANBURI THAILAND. NOV. 1977 - OCT. 1978

Appendix A.

STUDY NO.....

Name.....

Military Unit: Company.....Battalion..Division.....

Home Province.....Age....Rank.....Sex.....Race.....

Occupation: Before coming into the Army.....In the army.....

How long have you been in the Army?.....How long have you been stationed at

Pranburi?.....Where were you before (Changwat)?.....

(1) How many times have been admitted to the Hospital at Pranburi in the last year?...

For what reasons:.....Date.....

.....Date.....

.....Date.....

(2) Have been admitted to any other hospital in the last year?.....If so,

Where?.....

(3) Do you take any medicine regularly (including anti-malarials)?.....If so,

What?.....

(4) Have you ever had a blood transfusion?.....If so, when?.....

(5) Have you ever had hepatitis?.....If so, when?.....

(6) Have you had medical injections in the previous 12 mo?....If so, how many times.

(7) Have you had dental work in the previous 12 mo?....If so, how many times.....

(8) Have had a tattoo in the previous 12 mo?.....

(9) Non-wife.....(10) Razor use.....

(11) Have you donated blood in the previous 12 mo?.....If so, how many times.....

(12) Has anyone in your barracks had Jaundice in the previous 12 mo?....If so, how
many.....

(13) Has anyone in your Squad had Jaundice in the previous 12 mo?.....If so, how
many.....

(14) Has anyone in your Platoon had Jaundice in the previous 12 mo?.....If so, how
many.....

(15) Has anyone in your family had Jaundice in the previous 12.mo?.....If so,
how many.....

F. Continue to maintain a tight control on drug abuse. The highest hepatitis attack rates were in the unit with the highest drug abuse referral rate.

43. Studies on the Etiology of Acute Encephalitis in Patients at Bangkok Children's Hospital

OBJECTIVE: To determine the causative agent of encephalitis in patients admitted to Children's Hospital.

BACKGROUND: Encephalitis remains a major cause of morbidity and mortality among children in Thailand; the major known causes of the disease in this country include enteroviruses and Japanese Encephalitis Virus (JEV). Dengue viruses are generally thought to lack encephalitogenic potential in man; however, in mice and in monkeys certain strains of dengue can clearly grow well in brain tissue in vivo. This observation, coupled with scattered case reports from other countries of acute encephalitis in man due to dengue viruses, leave open the possibility that dengue viruses may under certain poorly understood circumstances be encephalitogenic in man.

Over the past several years cases of encephalitis have occurred frequently in children from within the greater metropolitan Bangkok area; in some of these cases a rise in anti-flavivirus HAI antibodies can be found. As dengue viruses are known to be endemic in greater metropolitan Bangkok and JEV is not, we investigated the possibility that occasional cases of encephalitis in metropolitan Bangkok were caused by dengue viruses.

METHODS: Beginning 1 May 1979 a detailed clinical history was recorded on patients with acute encephalitis admitted to Children's Hospital. Acute phase blood plasma and leukocytes, cerebro-spinal fluid, and autopsy materials (especially brain) were tested for isolation of arboviruses by standard tissue culture techniques in LLC-Mk2 cells.

Serological diagnosis of arboviruses infection acute and convalescent phase sera was performed by hemagglutination inhibition test using the standard micro-method.

RESULTS: Results of the first four months of the study are summarized in Tables 114-115. Sucrose density gradient fractionation is being done to clarify the diagnosis falling into the

Table 114 Serologic Diagnoses of Acute Encephalitis Cases

Diagnosis	# of patients with diagnosis	Case #
Definite JEV	3	E-002,003,004
Probable JEV	3	E-006,009,010
Unspecified Flavivirus	3	E-005,008,001
Other diagnosis (postmumps)	1	E-012
<u>Unknown</u>	<u>2</u>	E-007,011
Total	12	

Table 115 HAI Titers of Acute Encephalitis Patients Against Dengue and JEV Antigens.

Study No.	Age	Sex	Home	Day of Disease	HAI vs				JE
					D1	D2	D3	D4	
E001	11	F	Ayuthaya	12	1280	640	1280	1280	2560
				14	5120	640	1280	1280	1280
				28	5120	1280	2560	2560	5120
E002*	5	M	Supanburi	4	10	10	10	10	10
				6	10	10	10	10	20
				7	10	10	10	10	40
E003	4	M	Chachengsao	6	10	10	10	10	10
				22	10	10	10	20	40
E004*	3	F	Chachengsao	4	10	10	10	10	20
				6	10	10	10	10	160
				6	10	10	10	10	10
E005	8	F	Ayuthaya	8	640	1280	1280	5120	10240
E006*	9	M	Bangkok	15	80	80	160	160	160
E007	2	M	Smuthsakorn	4	10	10	20	80	20
				19	10	10	20	80	20
E008	9	F	Smuthprakarn	8	160	160	640	640	320
				25	1280	32	1280	1280	1280

Table 115 Continued

Study No.	Age	Sex Home	Day of Disease	HAI vs				
				D1	D2	D3	D4	JE
E009	4	M Smuthsakorn	6 23	10	10	20	40	640
				10	10	20	40	640
E010	12	F Petchaboon	6	10	10	10	10	20
E011*	3	F Chaiyapoom	60(HB1) (CSF)	20	10	40	20	10
				10	10	10	10	10
E012 (post- mumps)	4	M Bangkok	2	10	10	10	10	10
				10	10	10	10	10

* Fatal case

"Probable JEV" and "Unspecified Flavivirus" diagnostic categories. One definite virus isolate has been made, a JEV isolate from post-mortem brain tissue from case E-002.

This study will continue for a one year period.

44. Detection of Viruses in the Stools of Thai Children with Gastroenteritis by Electron Microscopy

OBJECTIVES:

1. To evaluate the frequency with which viruses can be visualized in stools of children with gastroenteritis in Bangkok.
2. To obtain serum and stool specimens from children with gastroenteritis to be used in the development and evaluation of assays for the diagnosis of viral gastroenteritis.

BACKGROUND: With the past 5 years a variety of viruses (rotaviruses, parvo-like viruses, non-cultivable adenoviruses) have been implicated as pathogens in acute non-bacterial gastroenteritis of humans (180). Evidence for infection with rotaviruses has been reported, in several large series, in over 40% to almost 90% of children with gastroenteritis (181-183). Although the overall role of viruses in gastroenteritis in adults is less well defined, both rotaviruses and parvo-like viruses appear to be well established as causes of acute outbreaks (184-187).

In 1976, workers at the IVR in Bangkok in collaboration with investigators from Ramathibodi Hospital detected rotaviruses in 39 of 100 stool specimens from small children by electron-microscopy (188). The results of this study were not published and no attempt was made to isolate and characterize the observed particles.

On the basis of preliminary cross-neutralizing antibody tests of rotavirus strains grown in LLC-Mk2 tissue cultures and detected by the fluorescent-focus method, Flewett et al (189) reported at least two and probably four serotypes of rotaviruses. Given the existence of multiple serotypes and the observation that pre-existing group-specific serum antibody is not protective, the level of immunity to rotaviruses in a community must be verified by neutralization of the locally prevalent serotypes. To do this, local strains of rotavirus must first be identified and collected.

Table 116 Virus-like Particles Visualized in Stool Specimens
from Bangkok Children with Gastroenteritis.

	Age (months)				Total
	0-5	6-11	12-17	18-24+	
Rotavirus	4	3	1	1	9
Adenovirus	1	0	0	1	2
? Enterovirus	0	1	0	0	1
Adenovirus + ? Parvovirus	1	0	0	0	1
No particles seen	14	9	3	5	31
	20	13	4	7	44

METHODS: Patients: Children less than two years old presenting to the Pramongkutklao Hospital or Children's Hospital clinics between 1 December 1978 and 1 April 1979 were candidates for study if they had gastroenteritis (nausea, vomiting, diarrhea) as judged by the hospital physician. No other restrictions were placed on entry into the study. Specifically, severity of disease was not a criterion.

Clinical specimens collected: The following were obtained from each patient: an acute blood specimen, an acute stool or diaper scraping, a 10-14 day convalescent blood, and a simple questionnaire.

Laboratory studies (Acute stool):

1. Routine culture for easily identified bacterial enteric pathogens (Salmonella and Shigella).

2. Electron Microscopy: The method of Zissis et al (190) was followed. Briefly, stool specimens were suspended about 30% (V/v) in PBS and centrifuged at 10,000 rev/minute for 30 minutes at 4°C in conical centrifuged tubes. The supernatants were again centrifuged at 10,000 rev/min for 30 minutes at 4°C. Five ml of the clarified supernatant were centrifuged at 250,000 g for one hour in a Beckman centrifuge (SW 50/rotor), and the pellet resuspended in 5 drops of distilled water. Electron-microscope grids, covered by a formular membrane, were placed on a drop of the suspension for 15 minutes, and the virus allowed to absorb to the membrane. After the membranes were dried, they were rinsed four times in a drop of saline, blotted dry after each dip, then negatively stained with 2% (v/v) uranyl acetate or sodium phosphotungstate. After drying, the grids were examined with the Hitachi (H-11C) electron-microscope at the IVR.

RESULTS Virus particles were detected in 13 of the 44 specimens examined by electron-microscopy. Table 116 summarizes the number of different morphologic types seen.

Of twelve specimens collected at the RTAH, none were positive for rotaviruses, one was positive for enterovirus-like particles, and one was positive for adenovirus. Of the 32 specimens collected at Children's Hospital, nine (28%) were positive for rotaviruses, one for adenovirus, and one for mixed adenovirus and parvovirus-like particles. The number of rotavirus particles visualized varied from < to more than 100 per grid square.

Table 117 Results of Serology of Serum Specimens from Department of Virology Personnel

Nationality	Name	HAV	Hep Bs	Any Alphavirus	CHIK	Any Flavivirus	D1	D2	D3	D4	JEV	Polio I	II	III
USA	Burke	-	-	-	0	-	0	0	0	0	0	+	+	+
	Watts	-	-	-	0	+	10	10	20	20	40	+	+	+
	Leach	+	-	-	0	-	0	0	0	0	0	+	+	+
Thai	Regina	-	-	-	0	-	0	0	0	0	0	+	+	+
	Ananda	+	-	+	160	+	40	40	80	160	160	+	+	+
	Rapin	+	+	+	160	+	20	40	40	160	40	+	+	+
	Naowayubol	+	+	+	320	+	20	20	40	160	40	+	+	-
	Chotampun	+	-	+	80	+	80	40	160	160	80	+	+	+
	Nathada	+	+	+	160	+	10	20	40	80	10	+	+	+
	Aree	+	+	+	160	+	80	80	320	320	160	+	+	+
	Pranee	+	+	+	0	+	80	40	80	320	160	+	+	+
	Somsamai	+	-	+	80	+	40	40	80	80	80	+	+	+
	Sanguan	+	+	+	2560	+	80	160	80	640	320	+	+	+
	Panor	+	+	+	2560	+	20	20	40	160	40	+	+	+
	Ming	+	+	+	80	+	80	40	80	160	80	+	+	+
	Pay	+	+	+	40	+	80	80	160	80	40	+	+	+
	Nongluk	+	-	+	80	+	40	40	80	80	80	+	+	-
	Somsak	+	+	+	160	+	20	40	40	160	80	+	+	+
	Pranom	+	+	+	160	+	20	20	20	40	20	+	+	+
	Anan	+	-	+	80	+	40	40	40	160	80	+	+	+
	Nongnard	+	-	+	320	+	20	40	20	40	80	+	+	+
	Sumitda	+	-	+	1280	+	40	40	40	160	80	+	+	+
	Boonmark	+	-	-	0	+	40	40	80	320	160	+	+	+

Table 118 Summary of Prevalence of Antibody to HAV, HBs, Alphavirus, Flaviviruses, and Polioviruses in Department of Virology Personnel.

Nationality	HAV	HBs	Any Alphavirus	Any Flavivirus	All 3 polio Types
U.S.	1/3	0/3	0/3	1/3	3/3
Thai	19/20	12/20	16/19	18/19	17/19
Total	20/23	12/23	16/22	19/22	20/22

45. Antibody Titers of Department of Virology Personnel to Selected Viruses

OBJECTIVE: To test serum specimens drawn from Department of Virology personnel for evidence of antibodies directed against pathogenic viruses currently used in the Department.

BACKGROUND: For years, research work in the Department of Virology has focused on flaviviruses and hepatitis B virus, with occasional work on other viruses including alphaviruses, specifically chikungunya, and polioviruses. Recently, work has begun on hepatitis A virus. As part of an overall effort to promote laboratory safety, serum specimens from department of personnel were tested for antibody against these viruses.

METHODS: A blood serum specimens was obtained from all department employees in January 1979. The following tests were performed:

<u>Activity measured</u>	<u>Test used</u>	<u>Criteria for positive assay</u>
Anti-hepatitis A virus	HAVAB(R) (SPRIA)	<9784 CPM
Hep BsAg	CIEOP	(+) precipitation line
Anti-HBs	AUSAB(R) (SPRIA)	>300 CPM
Anti-alpha virus	Chikungunya HAI	HAI titer $\geq 1/10$
Anti-flavivirus	Dengue-1,2,3,4 and JEV, HAI	HAI titer $\geq 1/10$
Anti-poliovirus	Microneut on Hela cells; metabolic inhibition end-point	Microneut titer $\geq 1/5$

RESULTS: Results are summarized in Table 117 and Table 118. All Thai employees except one had evidence of previous infections with both HAV and flaviviruses; this employee had spent her childhood in western Europe and the United States, and lacked serologic evidence of infection with either HAV or flavivirus.

One person, a known chronic carrier, was found to be positive for HBsAg (not shown in tables).

46. Investigation of the Epidemiology and Microbiology of Wounds and Wound Infections in the Royal Thai Army (RTA)

OBJECTIVES:

1. To describe the extent and distribution (by agent and anatomical site) of wounds, incurred as a result of combat, appearing at 1^o and 2^o surgical centers.
2. To establish the types and quantities of microbial flora coexisting with these injuries.
3. To relate microbial occurrence to risk of overt infection, severity and type in injury, subsequent treatment and residual morbidity.
4. To ascertain the value of the injury/microbiology/infection approach for use in predicting infection potential in combat injuries.

BACKGROUND: The pilot study and training period described previously demonstrated the feasibility of the establishment of a program to monitor care of an injured soldier through the chain of evacuation of the RTA.

Problems encountered in the establishment of this project, excepting those of study design and logistics, were mainly concerned with the gaining and holding of the interest of key personnel within the government and within the medical care system. For the practicing physicians, a continuations program of service was incorporated into the study design. For the government official, the potential long-term benefits in increased accuracy of prognosis and the eventual establishment of a hospital supported infection surveillance program were stressed.

METHODS: Study Sites: At the initiation of this study, the military hospital and the provincial hospital at Phitsanuloke were the focus of the chain of evacuation of patients injured near the Laotian border in Northeast Thailand. This changed during the period of study and therefore reduced the number of casualties, but did not change the distribution pattern of the injuries.

Pramongkutklao Hospital, Bangkok, was used as followup for patients enrolled at Phitsanuloke, and as a referral center for other hospitals in Thailand.

Microbiological Samples Collection: Both aerobic and anaerobic specimens were routinely collected from the wound site and a blood culture was taken concurrently. Wound specimens consisted of tissue,

fluid exudate or swab of infection surface, and were collected on admission, at surgery, and when clinically indicated. Wound edge and deep swabs were taken for comparison when a penetrating wound was evident. Cultures were inoculated into transport media and a blood culture bottle. Wound cultures were reported only when flora speciation and antibiotic sensitivity data was available. Blood specimens for culture were taken for culture on three successive days following any clinical indication of septicemia, following surgery, and following any positive culture report, and also taken if no specimen has been otherwise required for 3 days. Blood cultures were incubated for a minimum of 72 hrs., read at 24, 48, and 72 hrs., and then submitted to the main laboratory for further study. Any positive bottle culture was gram stained and examined microscopically prior to subculture.

Epidemiologic Data Collection: Each combat injured soldier was interviewed by a nurse as soon after admission as feasible. The interview concerned demographic data on the patient, type of injuring agent, if known (booby trap, mine, shoulder weapon, hand weapon, etc.), geographic location of the area in which the injury was sustained and activities at the time of injury. The nurse made an objective assessment of injury severity using a standard scale. From the patient's chart, a clinical description of all injuries and concurrent medical problems (diabetes, helminthiasis, etc.), therapy prior to admission, initial laboratory findings, and place and length of prior hospitalizations with this injury were abstracted.

RESULTS: Tables 119 - 120 present the demographic pattern of patients incurring combat injuries and subsequently requiring treatment at Pramongkutklao Hospital. The incidence of injury was heaviest on the lower ranking enlisted personnel in the 20-24 year old age group. The agent of injury, Table 121, was closely related to the anatomical location of the injury (Table 122). The extensive use of the land mine as an anti-personnel weapon by insurgent forces in Thailand has resulted in the high percentage of lower extremity injuries incurred.

Because extremity injuries predominated this series, further analysis will be specific for those type injuries. Similar analyses can and have been performed for other types of injuries.

Tables 123 and 124 present the bacterial isolates derived from extremity wounds. While enteric organisms, normally indigenous to man, were the predominating group, *Pseudomonas* species were isolated more often than any other single organism. Table 125

presents the length of hospitalization in lower extremity injuries in relation to the results of bacterial cultures taken at admission. Evidence of the presence of bacteria regardless of type, appeared to add approximately one month to the overall hospitalization in patients whose sole or primary injury was to the lower extremities. Antibiotic usage in these patients usually involved multiple drug therapy with combinations that included Ampicillin, Kanamycin and/or Penicillin G, Table 126. The antibiotic usage in patients from whom *Pseudomonas* sp. was isolated varied from that presented in Table 126 only in that there were fewer patients in which a great number of different antibiotics (over five) were used.

Of the 275 patients admitted to Pramongkutklao during the course of this study, 125 cases of lower extremity fracture and 43 upper extremity fractures were encountered. Often there were fractures of more than one extremity in an individual. Of the 125 lower extremity fractures (to include phalanges through femur), 92 (73.6%) eventually required some degree of amputation (Table 127). Eight upper extremity fractures (18.1% of the total) required ablative surgery. The majority of primary lower extremity amputations were traumatic. The extensive use of the land mine as an anti-personnel device is reflected here again. In spite of nearly universal evidence of infection on admission, non-amputated fractures arriving at Pramongkutklao sustained a less than 25% amputation rate in the lower extremity and an under 5% rate in the upper extremity.

The 78 patients identified at and followed from Phitsanuloke presented a chance to compare primary and secondary health care facilities. Because over half of the combat casualties at Phitsanuloke were civilian, the age range was much broader (8-50) and activities interrupted by combat were much less military in nature (farming, highway construction and hunting). Rifle bullets were the agents of injury in a higher percentage of cases in the primary center than the secondary, but injuries from land mines continued to predominate. There was a slightly less skewed array of injuries encountered at Phitsanuloke than at the secondary center in Bangkok, but lower extremity injuries were still over twice as common as any other anatomical location. Mean length of hospitalization was also considerable shorter in the outlying hospitals as compared to Pramongkutklao, 38 days vs 90+ days.

Bacterial isolate types from Phitsanuloke showed a very different distribution from that of Bangkok. While at Pramongkutklao,

TABLE 119

MILITARY STATUS OF COMBAT CASUALTY PATIENTS AT
PRAMONGKUTKLAO HOSPITAL, BANGKOK THAILAND 1978-9

	No.	%
Military and Police	223	
Private	130	58
NCO	79	36
Officer	14	6
Civilian	52	

TABLE 120
AGE OF COMBAT CASUALTY PATIENTS AT
PRAMONGKUTKLAO HOSPITAL, BANGKOK THAILAND 1978-9

Age	
<u><</u> 19	7
20-24	165
25-29	53
30-39	34
40-49	14
<u>></u> 50	2

TABLE 121

AGENT OF INJURY IN COMBAT CASUALTY PATIENTS AT
PRAMONGKUTKLAO HOSPITAL, BANGKOK THAILAND 1978-9

	No.	%
Land mine	123	42
Mortar, grenade	53	18
Rifle	105	36
Pistol	4	1
Artillery	2	1
Punji stick	1	-
Fire	1	-

* 16 cases received injuries
from 2 agents

TABLE 122

ANATOMICAL LOCATION OF INJURY IN COMBAT CASUALTY
PATIENT AT PRAMONGKUTKLAO HOSPITAL, BANGKOK THAILAND 1978-9

	No.	%
Head and neck	28	10
Thorax	34	12
Abd & genitalia	10	4
Upper extremity	46	17
Lower extremity	135	49
Multiple injuries involving more than one of the above classifications	22	8

TABLE 123
CULTURE RESULTS FROM LOWER EXTREMITY COMBAT INJURIES
SEEN AT PRAMONGKUTLAO HOSPITAL 1978-1979

	Lower Extremity (N=188)	Foot (N=32)
Pure Culture		
Pseudomonas sp.	40	5
Enteric organisms	44	5
Staphylococcus aureus	2	1
Mima sp.	3	
Bacillus subtilis	2	
Klebsiella sp.	2	
Candida		1
Skin flora	6	2
Mixed Culture		
Pseudomonas with enteric organisms	17	1
Pseudomonas with Staphylococcus	1	
Pseudomonas with skin flora	3	
Pseudomonas with enteric organisms and Staphylococcus	1	2
Pseudomonas with enteric organisms and Klebsiella	5	1
Enteric organisms with Staphylococcus	4	
Enteric organisms with Klebsiella	4	1
Enteric organisms with skin flora	2	1
Staphylococcus with Klebsiella	2	1
No Growth	50	11

TABLE 124

CULTURE RESULTS FROM COMBAT INJURIES OF THE UPPER
EXTREMITY SEEN AT PRAMONGKUTKLAO HOSPITAL (N=48)

Pure Culture

Pseudomonas sp.	7
Enteric organisms	5
Staphylococcus aureus	2
Mima sp.	2
Herellea sp.	1
Acinetobacter	1
Skin flora	1

Mixed Culture

Pseudomonas with staphylococcus	2
Pseudomonas with enteric organisms	1
Pseudomonas with skin flora	2
Pseudomonas with enterics and staphylococcus	1
Enterics with staphylococcus	1
Enterics with Klebsiella and streptococcus	1

No Bacterial Growth 28

TABLE 125
LENGTH OF HOSPITALIZATION IN LOWER EXTREMITY INJURIES
INCURRED AS A RESULT OF COMBAT BY BACTERIA ISOLATED ON ADMISSION

Organism (number of isolates)	Days of Hospitalization								Mean length of hospital- ization(days)
	<7	8-14	15-30	31-45	46-90	91-120	121-180	>180	
<i>Pseudomonas</i> sp. (67)	1		5	9*	8	11	8	25	132
<i>Enterobacter</i> sp. (51)	2*	1	3	3*	10	5	9	18	130
<i>Herellea</i> sp. (23)	1*	1	4	2*	1	1	5	8	125
<i>Klebsiella</i> sp. (20)				3		2	8	7	154
<i>Staphylococcus aureus</i> (16)			3	1	2	2	4	4	121
<i>Mima</i> sp. (3)				1	1			1	75
No bacterial growth (51)	2	4	7	5	10	4	10	9	98

* Pt. expired in 1 case

TABLE 126

ANTIBIOTIC THERAPY OF LOWER EXTREMITY
INJURY PATIENTS N=155

	No.	%
Ampicillin/Amoxicillin	108	70
Kanamycin	76	49
Penicillin G	60	39
Bactrim/Septrin	49	32
Gentamicin	30	19
Chloramphenicol	24	16
Cloxacillin	12	8
Tetracycline	7	5
Streptomycin	4	3
Lincomycin	2	1

TABLE 127

SEQUELLAE OF FRACTURES PRAMONGKUTKLAO HOSPITAL,
BANGKOK THAILAND 1978-1979

Fractures:

Lower Extremity (N=125)

Amputation prior to admission at Pramongkutklao Hospital	82(65.6%)
Amputation after admission to Pramongkutklao Hospital	10(8.0%)
Evidence of infection on admission in non-amputated fractures	33(76.7%)

Upper Extremity (N=43)

Amputation prior to admission at Pramongkutklao Hospital	6(13.9%)
Amputation after admission to Pramongkutklao Hospital	2(4.7%)
Evidence of infection on admission in non-amputated fractures	31(83.8%)

TABLE 128

AGREEMENT BETWEEN LOWER EXTREMITY WOUND CULTURE SAMPLES
PRAMONGKUTKLAO HOSPITAL, BANGKOK THAILAND

Organism	Deep and surface swabs agree	Deep and surface swabs disagree	Swabs and tissue agree	Swabs and tissue disagree
<i>Escherichiae coli</i>	5	3	1	2
<i>Enterobacter aerogenes</i>		2		2
<i>Enterobacter cloacae</i>	2	4	1	2
<i>Hereillea sp.</i>	2	3	1	1
<i>Klebsiella sp.</i>	2	3	1	5
<i>Pseudomonas sp.</i>	4	10	4	6
<i>Staphylococcus aureus</i>	1	5	1	1
<i>Streptococcus fecalis</i>		2	1	2

isolates of Pseudomonas sp. represented over one third of all isolates, at the primary health care center, Pseudomonas was identified in less than 10% of isolates. In non-pseudomonal isolates, Staphylococcus epidermidis and Enterobacter sp., especially E. agglomerans, represented a much higher percentage of wound associated isolates in the primary centers than in the secondary. Staphylococcus aureus was slightly more common in the secondary center. Antibiotic sensitivity patterns for bacteria isolated did not vary by level of care.

Three culture techniques were assessed as to agreement in bacterial isolation. Surface swabs agreed only occasionally with swabs taken from deep within the wound (Table 128). Except in the instance of the isolation of E. coli, given that an organism was isolated by one technique, in less than half of the cases could the organism be isolated from the other swab. The situation was similar for tissue specimens taken for culture. In no case was there more than 50% agreement between tissue specimen and swab specimen.

Data collection for this project is complete, but reduction and analysis will continue into the next year.

47. Hematologic Values in a Selected Normal Group of Bangkok School Children

OBJECTIVE: To determine the normal hematologic values on a select group of Bangkok students, of the ages of 6 to 9 years, accepted as clinically free of significant disease.

BACKGROUND: Clinical values in hematology, when reference normals are unavailable, are generally accepted as those presented by Wintrobe and others (191-194). Age differences for pediatric, adolescent, and adult groups have been presented and are generally accepted (193, 195, 196). Values for narrow age-range groups have been reported to be significantly different from standard literature references (197). This Laboratory has been studying a group of Bangkok school children, students at the Phibunprachasan School (196, 23). A requirement for definition of normal hematologic values with respect to these children provided the impetus for this study.

METHOD: The children, aged 6 to 9, are healthy Thai children participating in a longitudinal dengue hemorrhagic fever study. All are students of a lower middle class public school in Bangkok. Venous blood was drawn, preserved in disodium EDTA, and assayed within 7 hours of being drawn.

TABLE 122

STUDY VALUES

(E.L.: Extreme Low, M \pm I: Mean \pm I standard deviation, E. H.: Extreme High)

	Age		n		Age		n		Age		n	
	E. L.	M \pm I	E. H.	E. H.	E. L.	M \pm I	E. H.	E. H.	E. L.	M \pm I	E. L.	E. H.
TOTAL : n=40 Age : 6-9			6 7 8 9 10	10			6 7 8 9 10	5			6 7 8 9 10	5
					MALE : n=20 Age : 6-9		6 7 8 9 10	5			FEMALE : n=20 Age : 6-9	5
Height (in.)	38	46.4 \pm 2.9	50	38	45.7 \pm 3.2	50	38	41	47.0 \pm 2.5	49		
Weight (Kg)	15	19.6 \pm 2.6	25	15	19.0 \pm 2.5	24	15	15	20.2 \pm 2.7	25		
Hematocrit (%)	32	37.5 \pm 1.97	42	32	37.1 \pm 2.3	41	35	35	37.9 \pm 1.6	42		
Hemoglobin (gm)	10.6	12.4 \pm 0.87	14.3	10.6	12.3 \pm 0.9	13.7	11.0	11.0	12.5 \pm 0.8	14.3		
White blood cells (10^3)	5.1	8.96 \pm 2.48	15.2	5.8	8.71 \pm 2.07	12.5	5.1	5.1	9.20 \pm 2.88	15.2		
Sedimentation rate (mm/hr)	5	18 \pm 10	43	5	18 \pm 10	39	5	5	18 \pm 11	43		
Neutrophils (%)	23	47 \pm 11	68	25	51 \pm 12	68	23	23	43 \pm 10	54		
Band Neutrophils (%)	0	2 \pm 0.9	4	0	2 \pm 0.7	3	0	0	2 \pm 1.2	4		
Lymphocytes (%)	15	40 \pm 10	64	15	35 \pm 10	55	30	30	44 \pm 9	64		
Monocytes (%)	0	5 \pm 2	8	2	5 \pm 3	8	0	0	5 \pm 2	8		
Eosinophiles (%)	1	7 \pm 6	27	3	7 \pm 5	10	1	1	7 \pm 6	27		
Basophiles (%)	0	1 \pm 0.3	2	0	1 \pm 0.3	1	0	0	1 \pm 0.3	2		

SCHOOL CHILDREN, NORMAL POPULATION, PHIBUNPRACHASAN SCHOOL, 1979
DINDAENG AREA, BANGKOK, THAILAND

Table 130 Comparative Reference Values*

<u>Age 6-9(97%)</u>	<u>male</u>	<u>female</u>		
Height (in.)	49.7-57.2	49.4-56.5		
Weight (kg.)	27.7-40.8	26.6-40.8		
	<u>Age 6-10</u>	<u>Age 8-12</u>	<u>Age 21</u>	<u>Adult</u>
Hematocrit(%)		40		37-54
Hemoglobin(gm)		13.0-15.5		11-18
White blood cells(10^3)		8		5-10
Sedimentation rate(mm/hr)				0-15
Neutrophiles(%)	43-61		46-66	
Band neutrophiles(%)	6-11		5-11	
Lymphocytes(%)	42-48		24-44	
Monocytes(%)	4.3-4.7		4	
Eosinophiles(%)	2.4-2.7		2.7	
Basophiles(%)	0.5-0.6		0.5	

* IN: (1) Jacques Wallack. 1974. Interpretation of diagnostic test. 2nd. ed. p.4-7. Little, Brown and Co. Boston.

(2) Henry K. Silver, Kempe, C.H., and Bruyn, H.B. 1977. Hand Book of pediatrics, 12th. ed., p. 672-673. Lange Medical Publications, Los Altos, Calif.

Hematocrits were performed by micro-capillary tube centrifugation, hemoglobins measured spectrophotometrically by the Cyanmethemoglobin method using Drabkins solution, white blood cell counts by microscope and hemocytometer, sedimentation rates by the method of Wintrobe and Landsberg, and white blood cell differential counts by microscopic examination of Wright's stained thin film smears. The children's age, sex, height and weight was obtained during the blood drawing.

RESULTS: Table 129 shows the results of the study group and Table 130 provides comparison data from standard reference literature. Due to the small number sampled, tests of statistical significance were not performed and data is intended only to represent guideline information. There appears to be a difference in band neutrophile and eosinophile percentages. No explanation for the band neutrophile percentage difference is apparent. The eosinophila may be due to the endemicity of parasitic infections in Thailand. However, no efforts were made to confirm this and the children were considered normal based on history and inspection.

48. Support Activities of the Department of Veterinary Medicine

OBJECTIVE: To report the various activities of the Department of Veterinary Medicine during CY 79 (1 Oct 78-30 Sep 79).

RESULTS: Laboratory Animal Colony Activities - During FY 79, the laboratory animal colony supplied a large number of research animals to both AFRIMS investigators and investigators from several other medical schools and research institutions. The total number of animals bred, raised, conditioned and issued for use is summarized in Tables 131, 132 and 133. Table 134 lists the blood by species that was supplied to various investigators.

A total of 89 rhesus monkeys (Macama mulatta) were used in the antimalarial drug development program in FY 79. A total of 8 cynomolgus monkeys (Macaca fascicularis) were used in hepatitis and malaria projects during the year. A total of 15 cynomolgus monkeys were born between Jan 1979 and August 1979 in our cynomolgus breeding colony. This is just one short of a 100% birth rate in our first year of breeding this species. During the year we shipped 94 rhesus monkeys to the WRAIR for use by other investigators.

Table 131 Mouse Production. From October 78 - September 79

Number litters born		25,669
Number born		231,288
Weanlings Issued	AFRIMS	2,391
	Non-AFRIMS	34,163 (11 Institutions)
Adults Issued	AFRIMS	259
	Non-AFRIMS	1,271 (2 Institutions)
Litters Issued		6,846
Sucklings Issued	AFRIMS	10,775
	Non-AFRIMS	5,140 (1 Institution)
Total Deaths		3,217
Average pregnancy of female breeders		68 - 73%

Table 132 Hamster Production. October 78 - September 79.

Number litters born		42
Number born		339
Weanlings Issued	AFRIMS	0
	Non-AFRIMS	0
Adults Issued	AFRIMS	55
	Non-AFRIMS	158 (3 Institutions)
Total Deaths		47
Average pregnancy of female breeders		57 - 38%

Table 133 Guinea-pig Production. October 78 - September 79.

Number litters born		131
Number born		411
Weanlings Issued	AFRIMS	0
	Non-AFRIMS	448 (3 Institutions)
Adults Issued	AFRIMS	10
	Non-AFRIMS	14 (1 Institution)
Total Deaths		71

Table 134 Blood Issued. October 78 - September 79.

Sheep	AFRIMS	22,547 cc.
	Non-AFRIMS	9,550 cc. (4 Institutions)
Geese	AFRIMS	3,180 cc.
Rabbit	AFRIMS	1,230 cc.
Chicken	AFRIMS	250 cc.
Monkey	AFRIMS	200 cc.
Dog	AFRIMS	100 cc.
Cat	AFRIMS	100 cc.
Cow	AFRIMS	100 cc.
Pig	AFRIMS	100 cc.
Guinea-pig	AFRIMS	16 cc.
	Total	37,373 cc.

Table 135 Activities of the Hematology Laboratory for FY 79
1 October 78 - 30 September 79.

Malarial parasite Counts	6,610	Slides
Red Blood Cell Counts	886	Specimens
White Blood Cell Counts	1,485	Specimens
Drugs (weighed for malaria-infected monkeys)	1,801	doses
C.B.C. in Laboratory & Domestic Animals	235	Specimens
Fecal specimens examination for parasites	70	Specimens
Histopathology	877	Slides

During the year, we were able to establish a separate breeding colony of mice that would produce at least 200 suckling mice per day for use by the Department of Bacteriology and Clinical Laboratory Sciences in their E. coli toxin assay. This project requires mice 3-4 days old with body weights that fall within critical limits.

Hematology Laboratory Section: Activities of the hematology laboratory section during FY 79 are summarized in Table 135.

Three training courses in laboratory animal medicine were taught during FY 79. Two undergraduate courses, one for senior veterinary students from Kasetsart University lasting six hours and one for sophomore veterinary students from Chulalongkorn University lasting a total of 12 hours (6 hours lecture and 6 hours practical exercises) were taught. One post graduate training course for Mahidol University Master's degree candidates lasting a total of 12 hours was also taught.

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Work Unit 008 Tropical and Subtropical Diseases in Military
Medicine

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ²	2 DATE OF SUMMARY ²	REPORT CONTROL SYMBOL	
3 DATE OF SUMMARY ²		4 KIND OF SUMMARY	5 SUMMARY SCTY ²	6 WORK SECURITY ²	7 REGRADING ²	8 ITERN INSTR ²	9 DD-PHA (AR) 636
78 10 01		D. Change	U	U	NA	NL	DD-PHA (AR) 636
10 NO / CODES ²		PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		LEVEL OF SUM
A. PRIMARY		62770A	3M62770A802		00		A. WORK UNIT
B. CONTRIBUTING					009		
C. CONTRIBUTING		CARDS 114F					
11 TITLE (Precede with Security Classification Code) ²							
(U) Anti-Schistosomal Drug Development and Malaria Immunology and Vector Studies							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ²							
012600 Pharmacology 002600 Biology 010100 Microbiology							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
73 07		CONT		DA		C. In-house	
17 CONTRACT/GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE NA				PRECEDING		B. FUNDS (in thousands)	
B. NUMBER ²				FISCAL		79	
C. TYPE				YEAR		2.1	
D. KIND OF AWARD				CUMULATIVE		94	
E. CUM. AMT.				80		60	
20 RESPONSIBLE DOD ORGANIZATION				21 PERFORMING ORGANIZATION			
NAME ² Walter Reed Army Institute of Research				NAME ² US Army Medical Research Unit-Brasilia			
ADDRESS ² Washington, DC 20012				ADDRESS ² Brasilia, Brazil			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME. RUSSELL, Philip K., COL				NAME ² REID, WILLIS A., JR. LTC			
TELEPHONE. 202-576-3551				TELEPHONE: 272-4548 (Brazil)			
22 GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				ASSOCIATE INVESTIGATOR: ROBERTS, DONALD R. MAJ			
				NAME: McNEILL, K. MILLS, MAJ			
				NAME: PRATA, ALUIZIO R. MD			
23 KEYWORDS (Precede EACH with Security Classification Code)							
(U) Brazil; (U) Schistosomiasis; (U) Malaria; (U) Chemotherapy; (U) Immunology; (U) Epidemiology; (U) Drug Resistance; (U) Entomology							
24 TECHNICAL OBJECTIVE ² 25 APPROACH, 26 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code)							
23. (U) Find new prophylactic and curative drugs for the prevention and cure of schistosomiasis infections and to study the clinical, epidemiologic, drug susceptibility and vector transmission patterns of falciparum malaria in the Amazon River basin of Brazil. Both are primary diseases which would be acquired by U.S. Military and DOD civilian personnel in the event of deployment to any of numerous tropical areas of the world.							
24. (U) The WRAIR Anti-Schistosomal Drug Testing Program continues to submit candidate compounds for prophylactic (PMT) and curative (PCT) testing against schistosomiasis in mice. Compounds active in the primary screen are extensively reexamined for confirmation and dose response patterns. The malaria immunology studies include the testing of sera from endemic areas by the indirect fluorescent antibody test, in vitro drug susceptibility testing and creation of a cryobank of human strains of Plasmodium falciparum. Malaria vector transmission studies include field and laboratory analysis of morphological, behavioral, physiological and DDT susceptibility patterns of Anopheles darlingi and other potential anopheline malaria vectors.							
25. (U) 78 10 - 79 09. This research is complementary to studies being conducted under DAOB 6525, work Unit 086, entitled "Chemotherapeutic Studies on Schistosomiasis". During the reporting period 1065 compounds were screened in the PCT and PMT. Of these 58 were designated as confirmed or unconfirmed active and 232 were designated toxic. A malaria serological laboratory is now fully operational and providing logistical support for ongoing malaria field studies. Field collection, cryopreservation and in vitro cultivation and drug susceptibility testing of Plasmodium falciparum was accomplished. Complete entomological surveys were conducted at the field study sites. An. darlingi was found to be susceptible to DDT; movement, host seeking, biting activity and endophagic behavior was characterized. For technical report see Walter Reed Army Institute of Research Annual Report 1 Oct 78 - 30 Sep 79.							

PROJECT 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 009 Anti-schistosomal Drug Development

Investigators: Aluizio Rosa Prata, M.D.; LTC Willis A. Reid, Jr., MSC
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PART I. CHEMOTHERAPEUTIC STUDIES ON SCHISTOSOMIASIS.

1. Description: Schistosomiasis continues to be ranked as one of the most important of the tropical diseases, yet we still lack suitable means for chemotherapeutic management. The few drugs currently available demonstrate only partial curative efficacy and are often accompanied by adverse side effects ranging from carcinogenicity to headaches and dizziness. Considering the actual and potential global commitments of United States military and civilian personnel, the risks to infection with one of the human schistosomes remains high. Indeed the incidence of infection within foci of local indigenous populations may approach 100 percent. Consequently, a major research effort in anti-schistosomal drug development is being carried out by the Walter Reed Army Institute of Research (WRAIR) in conjunction with the University of Brasilia (USAMRU-Brasilia). The test compounds are obtained from the Division of Experimental Therapeutics (WRAIR) and are tested for prophylactic and/or curative activity in mice at preestablished dosage levels. The ultimate objective is the identification of compounds with a high potential for use in the prevention and treatment of schistosomiasis mansoni.

2. Progress:

a. Laboratory Facilities. At the beginning of the reporting period, the WRAIR program in Belém, Brazil (USAMRU-Belém) began the phase out of its operations. As a result of this closing, the USAMRU-Brasilia program acquired by transfer numerous items of equipment and laboratory and field supplies that have greatly enhanced the on-going schistosomiasis and malaria research efforts. Such key major items as microscopes, autoclave, vacuum pumps and centrifuge have been especially beneficial to the schistosomiasis program. Many expendable laboratory supplies were also acquired which significantly delayed the necessity to order for restocking current levels. Another major improvement in laboratory operations was the installation of a central air compressor in the Nucleo de

Medicina Tropical e Nutrição, thus providing a reliable source of aeration to the snail colony. Plans have also been submitted and approved for the construction of a small isolation room in the Pharmacy. A chemical exhaust hood will be installed in this area. These improvements will provide a safer area for the preparation of test drugs for anti-schistosomal therapy. We have also prepared a request for the purchase of an ultrasonic disintegrator system to be used in mixing compounds to assure an adequate drug suspension mixture in the designated vehicle. This will considerably improve the accuracy of drug dosage administration.

b. Animal Facilities. In FY78 we reported serious problems with the weekly supply of healthy mice from the University of Brasilia Bioterio (vivarium). The Bioterio has problems with rearing young mice to an acceptable weight (18-23 grams) and many of these are ill with a broad spectrum of parasitic, bacterial and/or viral infections. More recently, for example, there was an unusually high mortality of young mice from what appears to be ectromelia (mouse pox). In March and April of this year LTC Robert J. Beattie VC, Chief of the Department of Animal Resources, WRAIR, visited USAMRU-Brasilia at the request of the University of Brasilia. He engaged in extensive consultations with Dr. Andre de Mello, Chief of the UnB Bioterio, and they jointly published a report covering analyses of the problems, and recommended actions to bring the quality of animal management and facilities to acceptable standards. Those recommendations are now being studied by University officials and we hope soon to begin extensive physical renovations to the current facility. Such renovation should include at a minimum: 1) complete isolation of animal rooms from the outside environment to include separation of "clean" areas from "dirty" areas; 2) wash area with the provision of hot water and sterilization facilities; 3) control of air circulation, temperature and relative humidity in animal rooms; 4) adequate storage areas for food and bedding; and 5) reorganization of the personnel management structure for Bioterio animal technical personnel.

Current animal facilities within the drug testing laboratory are adequate for maintaining experimental mice. We continue to use ground corn-cob (cellulose) bedding material purchased annually from a local farmer. We are presently devising a method for improving this by filtering out the fine dust portion of the finished ground product.

c. Snail Colony. The Biomphalaria glabrata (Paulista Strain) snail colony continues to provide Schistosoma mansoni cercariae in sufficient quantities to perform the weekly mouse exposures for drug testing and life cycle maintenance. A weekly average of 400 snails were exposed to miracidia recovered from macerated

infected mouse livers. An unusually low level of prepatent mortality (5 percent) was obtained; of the survivors which were screened 42 days post-exposure, 57 percent were positive for emerging cercariae. These were maintained for future cercariae collections and a weekly average population of 1,200 positive snails (range 804 to 1,853) were on hand at one time. In general approximately 58 percent of the snails exposed were later recovered with patent infections.

d. Drug Testing. A total of 690 bottle number compound samples were received from WRAIR during the reporting period. Of these 5 compounds were predesignated for both prophylactic (PMT) and curative (PCT) testing; 557 compounds were predesignated for only curative testing and 128 compounds were predesignated for only mortality testing. Table 1 summarizes the workload data relative to drug testing. While 474 specific compounds were tested in the PCT, for example, 135 of those were compounds which were actually received during FY79. All others were tests of compounds received earlier and representing a backlog. The situation with the PMT testing backlog was more drastic. No compounds received during FY79 were actually tested during the reporting period; all compounds tested in the PMT were retests or tests of compounds received earlier than 1 Oct 78. The reasons for the accumulated backlogs are twofold: 1) increased emphasis on retesting compounds which show initial activity or toxicity, and 2) reduced numbers of mice available for weekly testing (see "Animal Facilities" above). Both factors worked to accumulate a backlog while compounds continued to be shipped from WRAIR. Additionally, many promising compounds were tested under "dose response" conditions at two routes of administration (subcutaneously or orally by gavage). Consequently, one compound test might involve the use of as many as 12 groups of test mice (70 mice). Indeed, one PMT run (PMT 78339) consisting of 250 test mice evaluated only 5 compounds under these conditions. All such retest backlogs have been eliminated and we are currently diminishing the untested drug backlog.

e. Personnel. The drug testing program is directed by one American Senior Investigator and supported by a staff of seven Brazilian Laboratory Assistants and one secretary/typist. The operating program is broken down into five work areas: 1) Snail Colony (two people); 2) Animal Service (one person); 3) Necropsy (one person); Pharmacy (two people); and Administration (two people). All individuals are cross-trained in procedures of snail maintenance, subcutaneous and gavage drug administration, daily mouse maintenance with mortality checks, and mouse exposures to cercariae. Each individual is able to perform all duties in at least two other areas of work. One of the persons listed under "Administration" is a

TABLE 1
FY78 workload data summary for USAMRU-Brasilia anti-shistosome drug testing program.

<u>Workload Criterion</u>	<u>PMT</u>	<u>PCT</u>	<u>Total</u>
<u>Number of Test Runs</u>	24	15	39
<u>Number of Untreated, Uninfected Control Groups*</u>	30	48	78
<u>Number of Untreated, Infected Control Groups*</u>	126	60	186
<u>Number of Reference Drug Groups*</u>	43	45	88
<u>Number of Test Drug Groups*</u>	849	595	1444
<u>Number of Drugs Tested (Total Bottle Number Compounds)....</u>	591	474	1065
<u>Number of Drugs Inactive and Non-Toxic.....</u> (See Tables and)	400	375	775
<u>Number of Drugs Toxic.....</u> (See Tables and)	173	59	232
<u>Number of Drugs Active (Confirmed or Unconfirmed)....</u> (See Tables and)	18	40	58
<u>Total Number of Mice Utilized</u>	NA	NA	13830
<u>Drug Testing</u>	5330	3650	8980
<u>Life Cycle</u>	NA	NA	3400
<u>Other</u>	NA	NA	1400

* One group contains 5 test mice.

senior laboratory technician capable of performing duties in all laboratory work areas as the need arises.

3. Test Procedures:

a. General: The current system of testing places a priority of prophylactic or curative testing on each compound. For those compounds received for testing in both systems, prophylactic testing is still performed first. All tests, prophylactic or curative, are performed with groups of five mice per drug per dosage. All mice are individually tail-exposed for 30 minutes to the numbers of cercariae required by the specific test. Drugs are routinely prepared for administration in a peanut oil vehicle unless another vehicle (such as water, saline, alcohol, or cremophor) has been previously recommended. All drugs are administered subcutaneously unless orally (by gavage) has been previously designated. Likewise, all drugs are administered in terms of mgs per kg body weight of mouse recipient.

b. Primary Mortality Test (PMT): The PMT is a prophylactic test in that it evaluates drug activity against immature migrating larval schistosomes. Mice are exposed to 3,000 - 3,500 S. mansoni cercariae. Two days after exposure, drugs are administered in a single inoculation to each of the five test animals per drug. The standard initial test dose is 640 mgs/kg and future testing may repeat this dose, with other groups being tested at lower dosages.

For every PMT group there are control groups of 1) 25 infected untreated mice, 2) 10 normal mice, and 3) five mice treated with the reference drug Niridazole (640 mgs/kg). The infected untreated control mice will begin dying on day 20 post-exposure and none will survive past day 30 in most cases. Niridazole-treated mice survive until day 49. Active drugs are those for which treated mice survive two weeks after the mean day of death of the infected control mice. At 49 days, all surviving mice (controls and drug test) are perfused for total worm burden determination (1). Drugs are considered toxic at the dosage given if recipient mice die within 10 days post-treatment (12 days post-exposure). All active compounds are scheduled for later retest confirmation at the same dosage and route of administration as the initial test. If positive confirmation is obtained for activity, then further testing at different dosages by both routes (subcutaneous or oral) is scheduled. Toxic compounds are retested at lower dosages until non-toxicity is obtained.

c. Primary Curative Test (PCT): The PCT is a curative test of a compound against an established S. mansoni infection in mice exposed to 160-200 cercariae. Thirty-three days post-exposure drugs

(100 mgs/kg) are administered daily for five consecutive days (until day 37) in the same manner as described for the PMT. Three days following the last treatment, all mice are: 1) killed individually by cervical dislocation; 2) the livers are immediately removed; 3) the livers are made into liver squash preparations; and 4) the numbers and condition of worms in the liver are determined for each surviving animal. Control groups for each PCT run are: 1) 20 untreated infection control mice; 2) 10 Niridazole treated mice (5 at 100 mgs/kg/day and 5 at 160 mgs/kg/day) and 3) five Oxamniquine treated mice (100 mgs/kg/day).

Criteria for drug activity are based upon the hepatic shift of adult worms from the mesenteries to the liver. This shift is presumed to be a result of drug pressure. Not only are the total numbers of liver worms determined but the conditions of those worms are also taken into consideration. The presence of dead worms is incontrovertable evidence of drug activity, while the presence of small, abnormally developed "sick" worms, possessing little movement, is evidence of possible activity requiring further testing.

Untreated control mice will normally show five to 15 worms (with a mean of 11 to 13 worms) in the liver. A mean test animal liver worm burden of 20 to 25 worms may be indicative of "marginal" drug activity. Such mean burdens higher than 25, even though the worms are living, is justification for retesting, possibly at a higher dosage level. Oxamniquine treatment produces high dead worm burdens in the livers of infected animals while Niridazole produces high living (but "sick") worm burdens at 100 mgs/kg and 160 mgs/kg, with the appearance of a few dead worms at the latter dose.

d. Secondary Curative Test (SCT): We reported earlier (Annual Technical Report for FY78) our efforts to expand the drug testing capabilities to secondary curative testing. At that time we were standardizing the test and formulating the data interpretation criteria. Those standardizations have been completed and we are prepared to initiate testing early in FY80.

The SCT is designed to determine the minimal dose of each drug which produces the death of virtually all worms and to characterize for each drug treatment the time required to produce an effect upon the residual live worm burden. The following effects will represent partial or complete drug activity: 1) the presence of dead worms and/or 2) the presence of abnormally low total worm burdens and/or 3) the presence of abnormally developed worms ("stunted", "sick" or otherwise representing abnormal morphologic development). This will be confirmed by more refined microscopic analysis. Table 2 depicts the proposed standard protocol for the SCT. As can be

seen from the numbers of animals required to test one drug at one dose, careful selection of candidate compounds must be made based upon prior results in the PCT in order to take maximum advantage of the test system.

Control infections in the SCT should behave as follows:

1) Infected, untreated controls: Total worm burdens (healthy worms) will remain constant throughout the analysis period (30% - 60% of cercarial exposure dose). There will be no or very few dead or abnormally developed worms present.

2) Infected, Niridazole-treated (160 mgs/kg/day X 5 days): There will be an increase in dead worm burden with time after treatment, from no or few dead worms on Day 3 post-treatment to greater than 90% dead worms on days 13 and 20 post-treatment. However, the total worm burden (dead + living worms) will remain high throughout the analysis period (Days 3 - 20 post-treatment).

3) Infected, treated with Oxamniquine (100 mgs/kg/day X 5 days): Greater than 90% of the worm burden will be killed by Day 3 post-treatment. The majority of the dead worms will be in the liver and will have undergone considerable deterioration (with concurrent dead worm granuloma formation) by day 20 post-treatment.

4. Results of Drug Testing. Tables 3 through 8 represent all drug testing results. A summarization of these results indicates that of the 591 PMT and 474 PCT compounds tested, 152 (25%) were PMT retests of previously tested drugs and 33 (7%) were PCT retests. Additionally 87 (15%) PMT compounds and 44 (9%) PCT compounds were tested twice during the reporting period because of toxicity or unconfirmed activity. Tables 3 and 6 identify those compounds which were reported as unconfirmed or confirmed actives in their respective tests. In the PMT, 18 compounds were reported as such (Table 3). All but three of these were in dose response tests, and all represent retests of compounds confirmed as active in previous years. Many of them, however, are now reported active in a wider range of dosages than previously reported. In the PCT, 40 compounds were reported as confirmed or unconfirmed actives (Table 6). Of these, 7 compounds represent PCT retests from previous years, 6 of which were tested under dose response conditions (see PMT tests above). All of the remaining 33 compounds were tested for the first time in FY79.

In the identification of active compounds, primary reference is made only to the bottle code numbers (and corresponding Brazil numbers) since many (but not all) of the compounds that we receive are protected proprietary secrets ("commercially discreet"). We

have, however, identified below the general classes of the more significant non-discreet active compounds. Numbers in parentheses represent the number of compounds of each class which showed indications of activity. For the PMT these are:

- heavy metals (5)
- quinoline methanols (3)
- nitro vinyl furan (1)
- nitro diphenyl isothiocyanate (1)

For the PCT these are:

- acridine (3)
- nitrofurans (2)
- heavy metals (2)
- piperazine (1)
- fluorene methanol (1)
- 8-aminoquinoline (1)
- quinoline methanol (1)
- phenanthrene methanol (1)

TABLE 2

Proposed protocol for the performance of the Secondary Curative Test (SCT) in the anti-schistosome drug development program of USAMRU-Brasilia.

<u>Day</u>	<u>Procedure</u>
0	Exposure all but 10 mice to 80-100 SmC
33	Divide all mice into the following groups: Uninfected/No Rx 10 mice Infected/No Rx 80 mice Infected/Rx Nirid. 160 ... 40 mice Infected/Rx Oxam 100 40 mice Infected/Rx Exp. Drug (40) mice/drug/dose/route *
33-37	Treat all mice (x mg/kg/day X 5 days)
40	Sacrifice $\frac{1}{4}$ of each group (Day 3 post-Rx)**
43	Sacrifice $\frac{1}{4}$ of each group (Day 6 post-Rx)**
50	Sacrifice $\frac{1}{4}$ of each group (Day 13 post-Rx)**
57	Sacrifice $\frac{1}{4}$ of each group (Day 20 post-Rx)**

*

The number of mice per test drug is to be determined on a drug-by-drug basis and is dependent upon the quantity of drug available. Under normal conditions use 40 mice per test drug group if sufficient drug quantities are available. Run as many experimental drug groups as the supply of mice permits.

**

Each mouse is to be killed by heparinized sodium pentobarbital, perfused and "liver pressed". Total worm burden will be recorded as "Living" and "Dead" and will be expressed as the sum of those perfused and those observed in the liver. Worms recovered will be killed, fixed and preserved in AFA for further analysis.

TABLE 3

Compounds examined in the Primary Mortality Test System (PMTS) against *S. mansoni* during FY79 and reported active at the test dosages indicated. The reported result in parentheses represents the number of mice surviving 14 days or more after the mean day of death of the untreated control mice compared to the total number of mice in the drug test group. An "unconfirmed" result represents activity in an initial test at the indicated dosage/route of administration but confirmation at the same dosage/route of administration has not yet been accomplished. "Test Run" is the Julian date on which the testing was initiated by mouse exposure to 3000 or more cercariae.

Brazil Number	Bottle Number	FY79 Test Run (Julian Date)	Drug Administration		Reported Test Result (Surviving Mice)	Confirmation
			Dosage (mg/kg)	Route		
00010	AG68873 =ZM27783	78339	160	SQ	Inactive (1/10)	NA
			320	SQ	Inactive (1/10)	NA
			640	SQ	Inactive (1/10)	NA
			1280	SQ	Active (5/10)	Unconfirmed
			1920	SQ	Active (3/5)	Confirms PMT 74289
00012 =00014 =00017	AY29559 =AY29568 =BB21481	78339	160	Gavage	Inactive (0/10)	NA
			320	Gavage	Inactive (1/10)	NA
			640	Gavage	Inactive (0/10)	NA
			1280	Gavage	Inactive (1/10)	NA
			20	SQ	Inactive (0/5)	NA
			40	SQ	Inactive (0/5)	NA
			80	SQ	Inactive (0/5)	NA
			160	SQ	Active (5/5)	Unconfirmed
			320	SQ	Active (5/5)	Unconfirmed (Prior confirmed Active at 640 and 1920 mg/kg)

TABLE 3 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>FY79 Test Run (Julian Date)</u>	<u>Drug Administration</u>		<u>Reported Test Result (Surviving Mice)</u>	<u>Confirmation</u>
			<u>Dosage (mg/kg)</u>	<u>Route</u>		
00012 =00014 =00017	AY29559 =AY29568 =BB21481	78339	20	Gavage	Inactive (0/5)	NA
			40	Gavage	Inactive (0/5)	NA
			80	Gavage	Inactive (0/5)	NA
			320	Gavage	Inactive (1/5)	NA
			640	Gavage	Inactive (0/5)	NA
00121	BB57310	78347	80	SQ	Active (3/5)	Unconfirmed (Prior confirmed Active at 640 and 1920 mg/kg)
01312 =04677	BE21931	78326	160	SQ	Inactive (0/5)	NA
			320	SQ	Inactive (0/5)	NA
			640	SQ	Inactive (1/5)	Confirmed Active in FY78
			1280	SQ	Active (4/5)	Unconfirmed
			160	Gavage	Inactive (0/5)	NA
			320	Gavage	Inactive (1/5)	NA
			640	Gavage	Inactive (0/5)	NA

TABLE 3 (continued)

Brazil Number	Bottle Number	FY79 Test Run (Julian Date)	Drug Administration		Reported Test Result (Surviving Mice)	Confirmation
			Dosage (mg/kg)	Route		
01313	BE21968	78347	20	SQ	Inactive (0/5)	NA
			40	SQ	Inactive (0/5)	NA
			80	SQ	Inactive (0/5)	NA
			160	SQ	Active (4/5)	Confirmed
			320	SQ	Active (5/5)	Confirmed
			640	SQ	Active (5/5)	Confirmed
			20	Gavage	Inactive (1/5)	NA
			40	Gavage	Inactive (1/5)	NA
			80	Gavage	Inactive (0/5)	NA
01317	AJ57633 =ZM33505	78354	160	Gavage	Inactive (0/5)	NA
			320	Gavage	Inactive (1/5)	NA
			640	Gavage	Active (5/5)	Unconfirmed
						Unconfirmed
			160	SQ	Inactive (0/5)	NA
			320	SQ	Inactive (1/5)	NA
			640	SQ	Active (5/5)	Confirms PMT 76091 and PMT 75064 (prior report)
			160	Gavage	Inactive (0/5)	NA
			320	Gavage	Inactive (0/5)	NA
			640	Gavage	Inactive (1/5)	NA

TABLE 3 (continued)

Brazil Number	Bottle Number	FY79 Test Run (Julian Date)	Drug Administration		Reported Test Result (Surviving Mice)	Confirmation
			Dosage (mg/kg)	Route		
01368 =04357	BE19575	78326	80	SQ	Inactive (1/5)	NA
			160	SQ	Active (4/5)	Confirms PMT 76217
			320	SQ	Toxic	NA
01567	BE13813 =ZN37106	78333	1280	SQ	Active (3/5)	Unconfirmed
01626 =04502	BG41577	78354	20	SQ	Inactive (0/5)	NA
			40	SQ	Inactive (0/5)	NA
			80	SQ	Inactive (0/5)	NA
			160	SQ	Active (5/5)	Unconfirmed
			320	SQ	Active (5/5)	Unconfirmed
			640	SQ	Active (3/5)	Unconfirmed
			1280	SQ	Active (5/5)	Unconfirmed
01674	BG43731 =ZN31953 =ZN80572	78347	20	Gavage	Inactive	NA
			40	Gavage	Inactive	NA
			80	Gavage	Inactive	NA
			160	Gavage	Inactive	NA
			320	Gavage	Inactive	NA
			640	Gavage	Inactive	NA
			1280	SQ	Active (4/5)	Confirms PMT 76126

TABLE 3 (continued)

Brazil Number	Bottle Number	FY79 Test Run (Julian Date)	Drug Administration		Reported Test Result (Surviving Mice)	Confirmation
			Dosage (mg/kg)	Route		
02560 =02909 =04709	AX94257 =BH58880 =ZN07500	78339	5	SQ	Inactive (1/5)	NA
			10	SQ	Inactive (0/5)	NA
			20	SQ	Inactive (0/5)	NA
			40	SQ	Inactive (0/5)	NA
			80	SQ	Inactive (1/5)	NA
			160	SQ	Active (5/5)	Confirms PMT 78116 and PMT 77145
02889	BH08111	79005	40	Gavage	Inactive (1/5)	NA
			160	Gavage	Active (4/5)	Unconfirmed
			640	Gavage	Active (5/5)	Unconfirmed
			40	SQ	Inactive (0/5)	NA
			80	SQ	Inactive (0/5)	NA
			160	SQ	Inactive (1/5)	NA
			320	SQ	Inactive (2/5)	NA
			640	SQ	Inactive (1/5)	Confirmed Active in FY78
			1280	SQ	Active (4/5)	Unconfirmed

TABLE 3 (continued)

Brazil Number	Bottle Number	FY79 Test Run (Julian Date)	Drug Administration		Reported Test Result (Surviving Mice)	Confirmation
			Dosage (mg/kg)	Route		
02889	BH08111	79005	40	Gavage	Inactive	NA
			80	Gavage	Inactive	NA
			160	Gavage	Inactive	NA
			320	Gavage	Inactive	NA
			640	Gavage	Inactive	NA
			1280	Gavage	Inactive	NA
02893	BH09157	79024	40	SQ	Inactive (0/5)	NA
			80	SQ	Inactive (2/5)	NA
			160	SQ	Active (4/5)	Unconfirmed
			640	SQ	Not Done	Confirmed Active in FY78
02894	BH08166	79024	40	Gavage	Inactive	NA
			80	Gavage	Inactive	NA
			160	Gavage	Inactive	NA
			40	SQ	Inactive (1/5)	NA
			80	SQ	Active (3/5)	Unconfirmed
			160	SQ	Inactive (1/5)	NA
			320	SQ	Active (4/5)	Unconfirmed
			640	SQ	Not Done	Confirmed Active in FY78

TABLE 3 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>FY79 Test Run (Julian Date)</u>	<u>Drug Administration</u>		<u>Reported Test Result (Surviving Mice)</u>	<u>Confirmation</u>
			<u>Dosage (mg/kg)</u>	<u>Route</u>		
02894	BH08166	79024	40	Gavage	Inactive	NA
			80	Gavage	Inactive	NA
			160	Gavage	Inactive	NA
			320	Gavage	Inactive	NA
02897	BH08200	79010	40	SQ	Inactive (0/5)	NA
			80	SQ	Inactive (1/5)	NA
			160	SQ	Inactive (2/5)	NA
			320	SQ	Active (3/5)	Unconfirmed
			640	SQ	Inactive (2/5)	Confirmed Active in FY78
			1280	SQ	Active (4/5)	Unconfirmed
			40	Gavage	Inactive (1/5)	NA
			80	Gavage	Inactive (0/5)	NA
			160	Gavage	Inactive (0/5)	NA
			320	Gavage	Inactive (0/5)	NA
			640	Gavage	Inactive (0/5)	NA
			1280	Gavage	Inactive (0/5)	NA

TABLE 3 (continued)

Brazil Number	Bottle Number	FY79 Test Run (Julian Date)	Drug Administration		Reported Test Result (Surviving Mice)	Confirmation
			Dosage (mg/kg)	Route		
02899	BH08228	79010	40	SQ	Inactive (0/5)	NA
			80	SQ	Inactive (0/5)	NA
			160	SQ	Inactive (1/5)	NA
			320	SQ	Active (4/5)	Unconfirmed
			640	SQ	Inactive (2/5)	Confirmed Active in FY78
			1280	SQ	Active (5/5)	Unconfirmed
			40	Gavage	Inactive	NA
			80	Gavage	Inactive	NA
			160	Gavage	Inactive	NA
			320	Gavage	Inactive	NA
			640	Gavage	Inactive	NA
			1280	Gavage	Inactive	NA
03809	BH30033	79017	1280	SQ	Not Done	Confirmed Active in FY78
			10	Gavage	Inactive (0/5)	NA
			40	Gavage	Inactive (0/5)	NA
			80	Gavage	Inactive (2/5)	NA
			160	Gavage	Inactive (1/5)	NA
			320	Gavage	Inactive (1/5)	NA
			640	Gavage	Active (5/5)	Unconfirmed
			1280	Gavage	Active (3/5)	Unconfirmed

TABLE 4

Compounds screened in the Primary Mortality Test System (PMTS) against S. mansoni during FY79 and determined to be toxic (T) at the test dosages indicated. Repetition of the same dose indicates that the compound was retested for confirmation. The lack of a toxicity indicator (T) represents non-toxicity and inactivity at that dosage. All drugs were administered subcutaneously.

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
00315	BB71132	640 T	01461	BE67571	1280 T
00516	BC21600	40 80 160 T	01525	BB43996	1280 T
00959	BB91563	20 40 80 160 320 T	01818	BG43937	40 80 160 T
00962	BB91616	120 320 640 T	02040	BG47775	1280 T
00116	BE43786	320 T	02157	BG60189	40 T
01334	BE67651	640 T	02245	BG60161	40 T
01432	BE70881	1280 T	02246	BG62807	40 T
			02307	BE98076	80 T
			02421	BG69100	160 T 320 T

TABLE 4 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
02452	BG72590	40 T	03204	BC39620	320 640 T
02470	BG80574	40 T			
02923	BH09323	20 T	03206	BC39853	320 T 640 T
02924	BH09332	20 T	03208	BC39979	320 T 640 T
02963	BH12964	20 T			
02990	BH14360	160 T	03209	BC45904	320 T 640 T
03038	AT90777	640 1280 T	03226	BB65607	320 640 T
03066	BH13916	640 T	03228	BC10509	320 T 640 T
03069	BH16613	160 T			
03070	BH16711	160 T	03229	BC11239	320 T 640 T
03199	BC39344	320 T 640 T	03230	BC13073	320 T 640 T

TABLE 4 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
03235	BC30690	320 T 640 T	03256	BC35220	160 640 T
03236	BC30805	640 T	03260	BC39139	160 T 640 T
03240	BC31615	640 T	03267	BE39755	160 640 T
03241	BC31928	640 T	03273	BB66051	160 640 T
03243	BC32078	640 T	03274	BB66113	160 T 640 T
03244	BC32176	640 T	03276	BC11328	160 640 T
03246	BC32498	640 T	03277	BC14794	160 T 640 T 640 T
03248	BC32532	640 T	03279	BC15577	160 640 T
03249	BC32578	640 T			
03250	BC32587	640 T			
03251	BC32701	640 T			
03252	BC32890	160 640 T			

TABLE 4 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
03280	BC16118	160 640 T	03302	BC32765	80 640 T
03282	BC30627	160 240 T	03304	BC33299	80 640 T
03287	BC31777	160 640 T	03305	BC33404	160 640 T
03289	BC31964	160 T 640 T	03307	BC33477	80 640 T
03293	BC32096	320 640 T	03308	BC33486	80 640 T
03296	BC32185	80 640 T	03310	BC34063	80 640 T
03299	BC32541	80 640 T	03311	BC34072	80 640 T
03300	BC32550	80 640 T	03312	BC34090	80 640 T

TABLE 4 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
03313	BC34125	80 640 T	03327	BC34778	80 T 640 T
03318	BH23930	80 640 T	03329	BC35426	80 640 T
03319	BH23949	80 640 T	03331	BC35855	80 640 T
03320	BH23958	80 640 T	03334	BC36521	86 640 T
03321	BH23967	80 640 T	03336	BC79066	80 T 640 T
03323	BH23985	80 640 T	03337	BC37608	80 T 640 T
03325	BH24008	80 640 T	03338	BC37751	80 T 640 T
03326	BH24017	80 640 T	03341	BC38105	80 T 640 T

TABLE 4 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
03342	BC38123	80 T 640 T	03372	BH17236	80 T 640 T
03343	BC38392	80 640 T	03373	BH17245	80 T 640 T
03344	BC38918	80 T 640 T	03375	BB66211	320 640 T
03348	BC63779	160 640 T	03378	BC10189	320 640 T
03349	BD85056	80 640 T	03380	BC10483	320 640 T
03350	BD85501	80 640 T			
03354	BD99069	160 640 T	03382	BC16252	160 T 640 T
03356	BE64230	160 640 T	03386	BC31099	320 T 640 T 640 T
03370	BH17218	80 T 640 T			

TABLE 4 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
03397	BC33217	320 640 T	03414	BC39442	160 640 T
03398	BC33459	160 T 640 T	03415	BC39602	160 640 T
03400	BC33619	160 640 T	03416	BC39960	320 640 T
03401	BC33708	320 640 T	03421	BD93996	160 T 640 T
03404	BC34643	160 640 T	03426	BB65483	160 640 T
03406	BC35033	320 640 T	03434	BC10821	160 T 640 T
03408	BC35837	160 T 640 T	03440	BC11060	640 T
03409	BC36692	160 640 T	03447	BC11266	640 T
			03453	BC11524	640 T

TABLE 4 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
03466	BC12030	640 T	03551	BG19066	640 T
03479	BC12281	640 T	03552	BH29852	640 T
03489	BC15540	640 T	03553	BH29898	640 T
03494	BC31053	640 T	03560	BG10223	640 T
03500	BC34625	640 T	03561	BG10581	640 T
03506	BH17281	640 T	03565	BG16430	640 T
03517	BC11140	640 T	03566	BG16565	640 T
03522	BC11480	640 T	03574	BG17820	640 T
03537	BC11818	640 T	03577	BG17868	640 T
03542	BG12405	640 T	03578	BG18005	640 T
03543	BG12441	640 T	03580	BG18578	640 T
03548	BG15433	640 T	03581	BG18738	640 T
03550	BG18649	640 T	03582	BG18818	640 T

TABLE 4 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
03584	BG18836	640 T	03604	BC38249	640 T
03585	BG18907	640 T	03608	AY99588	640 T
03586	BG18952	640 T	03610	BB41830	640 T
03588	BG19075	640 T	03611	BB41849	640 T
03589	BC19128	640 T	03632	BC36825	640 T
03590	BC19637	640 T	03636	BC10198	640 T
03591	BC19646	640 T	03637	BC10250	640 T
03592	BC19655	640 T	03640	BC10385	640 T
03593	BC19682	640 T	03647	BC10607	640 T
03595	BC19708	640 T	03649	BC10689	640 T
03599	BC33262	640 T	03651	BC10705	640 T
03600	BC34803	640 T	03652	BC10750	640 T
03601	BC36165	640 T	03665	BC13224	640 T

TABLE 4 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
04405	BG81679	640 1280 T

TABLE 5

Compounds screened in the Primary Mortality Test System (PMTS) against S. mansoni during FY79 and determined to be inactive and non-toxic at the test dosages indicated. All compounds were administered subcutaneously.

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
00033	BB89821	160	00334	BB71534	640
00206	BB67414	160	00336	BB71561	640
00306	BB70948	640	00337	BB71570	640
00307	BB70957	640	00338	BB71598	640
00310	BB71061	640	00340	BB71641	640
00318	BB71187	640	00341	BB71669	640
00322	BB71258	640	00345	BB88708	640
00328	BB71409	640	00346	BB38717	640
00329	BB71418	640	00348	BB88735	640
00332	BB71454	640	00349	BB88744	320
00333	BB71507	640	00356	BB88851	640

TABLE 5 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
00359	BB88888	160	00923	BB88093	320
00361	BB88922	640	00935	BB88280	640
00363	BB89009	640 1280 1920	01011	BB92695	160 320
00365	BB70822	160	01021	BE17615	1280
00424	BE15148	640	01118	BE43802	160
00563	BC21628	640	01178	BB47725	1280
00625	BD68340	640	01183	BB47903	1280
00631	BB44484	640	01215	BC52874	1280
00782	BC21253	640	01266	AV13275	320
00821	BC26794	640	01321	BE57646	80 160 320
00891	BB74320	80 160			

TABLE 5 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
01336	BE67679	40 80 160	01520	BG09293	1280
01340	BE67731	1280	01628	EG44014	1280
01387	BE70390	1280	01630	AY98670	640 1280 1920
01392	BE70470	1280	01681	AV99065	1280
01394	BE70505	1280	01698	BE97211	1280
01407	BE70783	1280	01701	BE97300	1280
01408	BE70792	1280	01714	BG41086	80 160 320
01462	BE70658	1280			
01489	BE18710	1280	01721	BE97426	1280
01491	BE18747	1280	01816	BG39684	40 80 160
01493	BE18774	1280			
01519	BG09284	1280	01865	BE97891	640

TABLE 5 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
01883	BG39693	320	02329	AK23525	80
02040	BG47686	80	02372	BG39415	80
		160	02379	BG39997	80
		320	02393	BG40963	80
02054	BG47855	1280	02396	BG58689	80
02263	BG68443	40	02400	BG59248	40
02266	BG70729	40	02426	BG69431	80
02267	BG70756	40	02433	BG69860	40
02281	AY46050	80	02445	AV37127	160 320
02301	BE93017	40	02447	BC82023	40
02302	BE97837	40	02456	BG75064	1280
02304	BE97926	320	02471	BG80618	80
02312	BE98192	40			
02324	AG56396	40			

TABLE 5 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
02472	BG80672	40	02900	BH08237	20
02492	BG81124	1280	02901	BH08246	40
02664	BC15273	1280	02911	BH09181	640
02672	BC15460	640 1280	02918	BH09261	20
02844	BH05727	40	02919	BH09270	40
02865	BH09387	20	02922	BH09314	40
02872	BH07936	40	02951	BH10317	20
02874	BH07954	20	02956	BH09921	20
02875	BH07963	20	02959	BH09985	20
02876	BH07972	40	02996	AF92511	320
02877	BH07981	40	03006	AV58373	160
02878	BH07990	20	03016	AE07642	160
			03048	AX29054	160

TABLE 5 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
03052	AY72309	160	03203	BC39611	640
03053	AY72407	160	03205	BC39808	640
03174	AU65162	640 960	03207	BC39899	640
03177	AU65966	640 960	03210	BD52486	640
03194	BC39228	640	03211	BE82621	640
03195	BC39282	640	03212	BE98156	640
03196	BC39291	640	03213	BH16560	640
03197	BC39308	640	03219	ZM34333	640
03198	BC39326	640	03220	BH16677	640
03200	BC39522	640	03221	BH16686	640
03201	BC39531	640	03222	BH16695	640
03202	BC39540	640	03223	BH16702	640
			03224	BH16828	640

TABLE 5 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
03225	BB65410	640	03257	BC35239	640
03227	BC10134	640	03258	BC36530	640
03232	BC16261	640	03259	BC37895	640
03233	BC16298	640	03261	BC39193	640
03234	BC30645	640	03262	BC39424	640
03237	BC30841	640	03263	BC39764	640
03238	BC30850	640	03264	BC39773	640
03239	BC31446	640	03265	BD85216	640
03242	BC32041	640	03266	BD88762	640
03245	BC32363	640	03268	BE40294	640
03253	BC32916	640	03269	BE64221	640
03254	BC33771	640	03270	BE64605	640
03255	BC34714	640	03271	BE64758	640

TABLE 5 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
03272	B865714	640	03295	BC32130	80 640
03275	BC10867	640	03297	BC32194	640
03278	BC15424	640	03298	BC32201	640
03281	BC30038	640	03301	BC32685	640
03283	BC31035	640	03303	BC32907	640
03284	BC31400	640	03306	BC33440	640
03285	BC31633	640	03309	BC33566	640
03286	BC31713	640	03314	BC34563	640
03288	BC31919	640	03315	BC34572	640
03290	BC31973	640	03316	BC34705	640
03291	BC31982	640	03317	BC23921	640
03292	BC31991	640	03322	BH23976	640
03294	BC32112	640			

TABLE 5 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
03324	BH23994	640	03353	BD91821	640
03328	BC35248	640	03355	BE39442	640
03330	BC35499	640	03357	BH16971	640
03332	BC36138	640	03358	BH17030	640
03333	BC36370	640	03359	BH17058	640
03335	BC37117	640	03360	BH17067	640
03339	BC37902	640	03361	BH17076	640
03340	BC37966	640	03362	BH17094	640
03345	BC39077	640	03363	BH17101	640
03346	BC39120	640	03364	BH17110	640
03347	BC39826	640	03365	BH17129	640
03351	BD88735	640	03366	BH17138	640
03352	BD90235	640	03367	BH17147	640

TABLE 5 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
03368	BH17165	640	03388	BC31508	640
03369	BH17209	640	03389	BC32167	640
03371	BH17227	640	03390	BC32309	640
03374	BB65938	640	03391	BC32452	640
03376	BC10063	640	03392	BC32676	640
03377	BC10152	640	03393	BC32694	640 960
03379	BC10438	640	03394	BC32729	640
03381	BC16136	640	03395	BC32756	640
03383	BC19548	640	03396	BC32836	640
03384	BC30163	640 960	03399	BC33502	640
03385	BC30789	640	03402	BC33735	640
03387	BC31124	640	03403	BC33879	640

TABLE 5 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
03402	BC33735	640	03424	ZN37704	640
03403	BC33879	640	03425	BB62802	640 960
03405	BC34750	640	03427	BB65625	640
03407	BC35202	640	03428	BB65830	640
03410	BC36709	640	03429	BB65965	640
03411	BC37555	640	03430	BB65983	640
03412	BC38767	640	03431	BB66060	640
03413	BC39157	640	03432	BB66239	640
03417	BC63537	640 960	03433	BB91812	640
03418	BC63715	640	03435	BC10830	640
03419	BD85903	640	03436	BC10885	640
03420	BD90691	640	03437	BC10947	640
03422	BH26986	640	03438	BC10965	640

TABLE 5 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
03439	BC11006	640	03458	BC11640	640
03442	BC11159	640	03460	BC11702	640
03444	BC11195	640	03461	BC11837	640
03445	BC11202	640	03463	BC11917	640
03446	BC11257	640	03464	BC11935	640
03448	BC11300	640	03465	BC12003	640
03450	BC11355	640	03467	BC12058	640
03451	BC11471	640	03469	BC12110	640
03454	BC11533	640	03470	BC12129	640
03455	BC11542	640	03471	BC12138	640
03456	BC11597	640	03472	BC12156	640
03457	BC11631	640	03473	BC12165	640

TABLE 5 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
03474	BC12209	640	03492	BC15675	640
03475	BC12218	640	03493	BC15906	640
03476	BC12227	640	03495	BC32069	640
03477	BC12236	640	03496	BC33682	640
03478	BC12263	640	03497	BC33691	640
03480	BC12290	640	03498	BC33977	640
03481	BC12343	640	03499	BC43223	640
03485	BC15362	640	03501	BC38178	640
03486	BC15479	640	03502	BC39273	640
03487	BC15504	640	03503	BC63653	640
03488	BC15522	640	03504	BH17156	640
03490	BC15602	640	03505	BH17174	640
03491	BC15648	640	03507	ZN38489	640

TABLE 5 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
03508	ZN39262	640	03526	BC11588	640
03509	BH17049	640	03529	BC11677	640
03511	BC10849	640	03530	BC11686	640
03512	BC10901	640	03531	BC11720	640
03513	BC10929	640	03532	BC11739	640
03514	BC10974	640	03533	BC11748	640
03515	BC11015	640	03534	BC11766	640
03516	BC11122	640	03536	BC11800	640
03519	BC11280	640	03538	BC11828	640
03520	BC11364	640	03539	BC11891	640
03521	BC11444	640	03540	BC11908	640
03523	BC11499	640	03541	BC11980	640
03525	BC11560	640	03545	BG12478	640

TABLE 5 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
03546	BG15228	640	03568	BG17008	640
03547	BG15317	640	03569	BG17133	640
03549	BG15835	640	03570	BG17320	640
03554	BH29914	640	03571	BG17508	640
03555	BH29941	640	03572	BG17553	640
03556	BH29950	640	03573	BG17562	640
03557	BH29987	640	03575	BG17839	640
03558	BH30346	160	03576	BG17857	640
03559	BH30355	160	03579	BG18470	640
03562	BG12254	640	03583	BG18827	640
03563	BG14203	640	03587	BC19039	640
03564	BG15353	640	03594	BC19691	640
03567	BG16994	640	03597	BC30029	640

TABLE 5 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
03598	BC31179	640	03642	BC10518	640
03602	BC36245	640	03643	BC10536	640
03603	BC36496	640	03644	BC10554	640
03605	BC38365	640	03645	BC10563	640
03606	BC39513	640	03646	BC10572	640
03607	BC39791	640	03648	BC10616	640
03609	BB40422	640	03650	BC10698	640
03631	BC35711	640	03653	BC10803	640
03633	BC10090	640	03654	BC10983	640
03634	BC10107	640	03656	BC11051	640
03638	BC10269	640	03657	BC11113	640
03639	BC10278	640	03658	BC11248	640
03641	BC10492	640			

TABLE 5 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
03659	BC11319	640
03660	BC11346	640
03661	BC11373	640
03662	BC11426	640
03663	BC11579	640
03664	BC13162	640
04647	BH56608	320 640

TABLE 6

Compounds examined in the Primary Curative Test (PCT) System against *S. mansoni* during FY79 and reported active in one or more tests at the test dosages indicated. Numbers in parentheses represent the mean number of worms in the livers of surviving treated mice. A compound is considered active in the PCT if mean liver worm burdens reach 20 or more; a mean of 20 to 25 worms represents "marginal" (M) activity. An "unconfirmed" result represents activity in an initial test at the indicated dosage/route of administration but confirmation at the same dosage/route of administration had not yet been accomplished. "Test run" is the Julian date on which the testing was initiated by mouse exposure to approximately 200 (+ 10%) cercariae.

Brazil Number	Bottle Number	FY79 Test Run (Julian Date)	Drug Administration		Reported Test Result (Mean No. Worms/Liver)	Confirmation
			Dosage (mg/kg)	Route		
01179	BB47734	79031	160	Gavage	Active (28) Toxic	Confirms PCT 76329 NA
			320	Gavage		
01203 =04666	BC07271	79031	40	Gavage	Inactive (12)	NA
			80	Gavage	Inactive (14)	NA
			160	Gavage	Inactive (19)	NA
			320	Gavage	Active (50)	Confirms PCT 78144
			640	Gavage	Active (45)	Unconfirmed
01533 =05104	BC42725 =BH73209	79031	5	SQ	Inactive (17)	NA
			10	SQ	Inactive (17)	NA
			20	SQ	Active (21)(M)	Unconfirmed
			40	SQ	Not Done	Confirmed Active in FY78

TABLE 6 (continued)

Brazil Number	Bottle Number	FY79 Test Run (JULIAN Date)	Drug Administration		Reported Test Result (Mean No. Worms/Liver)	Confirmation
			Dosage (mg/kg)	Route		
01617	BG32514	79031	10	SQ	Inactive (15)	NA
			20	SQ	Active (22)(M)	Unconfirmed
			40	SQ	Toxic	NA
02456	BG75064	79031	80	Gavage	Active (33)	Unconfirmed Prior confirmed activity at 100 and 200 mg/kg (SQ) reported in FY78
02457	BG75073	79031	80	Gavage	Active (23)(M)	Unconfirmed
			160	Gavage	Active (40)	Unconfirmed
02685 =03508	BB69329 ZN39262	79031	5	SQ	Inactive (13)	NA
			10	SQ	Inactive (15)	NA
			20	SQ	Inactive (18)	NA
			40	SQ	Active (26)	Confirmed
			80	SQ	Active (33)	Unconfirmed
			160	SQ	Active (17; 20% dead)	Unconfirmed
			5	Gavage	Inactive (15)	NA
			10	Gavage	Toxic	NA
			20	Gavage	Inactive (16)	NA
			40	Gavage	Inactive (16)	NA
			80	Gavage	Active (27)	Unconfirmed
			160	Gavage	Active (29)	Unconfirmed

TABLE 6 (continued)

Brazil Number	Bottle Number	FY79 Test R:n (Julian Date)	Drug Administration		Reported Test Result (Mean No. Worms/Liver)	Confirmation
			Dosage (mg/kg)	Route		
02882	BH08040	79031	40	SQ	Active (19, 90% with abnormal development)	Unconfirmed
04800	AB10813	78277	100	SQ	Active (21)(M)	Unconfirmed (marginal)
04804	AB16253	79031	80	SQ	Inactive (10)	NA
			160	SQ	Inactive (10)	NA
		78277	100	SQ	Active (20)(M)	Unconfirmed (marginal)
04807	AB27363	79031	80	SQ	Inactive (8)	NA
			100	SQ	Inactive (7)	Fails to confirm marginal activity in 78277
		78277	100	SQ	Toxic	NA
		79045	40	SQ	Active (22)(M)	Unconfirmed
			80	SQ	Toxic	NA
		79143	20	SQ	Inactive (10)	NA
			40	SQ	Inactive (11)	Fails to confirm activity in PCT 79045. Testing terminated.

TABLE 6 (continued)

Brazil Number	Bottle Number	FY79 Test Run (Julian Date)	Drug Administration		Reported Test Result (Mean No. Worms/Liver)	Confirmation
			Dosage (mg/kg)	Route		
04813	AB55769	78277	100	SQ	Active (20)(M)	Unconfirmed
		79031	160	SQ	Inactive (13)	Fails to confirm activity at a lower dose in PCT 78277. Testing terminated.
04833	AB88777	79052	100	SQ	Active (20)(M)	Unconfirmed
		79122	50	SQ	Inactive (18)	NA
			100	SQ	Active (20)(M)	Confirms marginal activity of PCT 79052
04899	AD44953	79122	100	SQ	Active (20)(M)	Unconfirmed
		79157	100	SQ	Inactive (15)	Fails to confirm marginal activity of PCT 79122. Testing terminated.
04900	AD45772	79073	100	SQ	Toxic	NA

TABLE 6 (continued)

Brazil Number	Bottle Number	FY79 Test Run (Julian Date)	Drug Administration		Reported Test Result (Mean No. Worms/Liver)	Confirmation
			Dosage (mg/kg)	Route		
04900	AD45772	79122	50	SQ	Toxic	NA
		79157	10 20	SQ SQ	Inactive (11) Active (26)	NA Unconfirmed
04939	AE00634	79087	100	SQ	Toxic	NA
		79122	50	SQ	Toxic	NA
04954	AE12769	79157	10 20	SQ SQ	Active (20)(M) Active (21)(M)	Unconfirmed Unconfirmed
		79101	100	SQ	Active (27)	Unconfirmed
04955	AE14389	79122	50 100	SQ SQ	Inactive (18) Active (20)(M)	NA Confirms PCT 78101
		79157	80	SQ	Active (24)(M)	Unconfirmed
		79101	100	SQ	Active (22)(M)	Unconfirmed
		79122	50 100	SQ SQ	Inactive (17) Inactive (16)	NA Fails to confirm marginal activity of PCT 79101. Testing terminated.

TABLE 6 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>FY79 Test Run (Julian Date)</u>	<u>Drug Administration</u>		<u>Reported Test Result (Mean No. Worms/Liver)</u>	<u>Confirmation</u>
			<u>Dosage (mg/kg)</u>	<u>Route</u>		
04970	AE43522	79101	100	SQ	Active (21)(M)	Unconfirmed
05044	AD08402	79108	100	SQ	Active (22)(M)	Unconfirmed
05068	AD32695	79122	100	SQ	Active (20)(M)	Unconfirmed
		79157	100	SQ	Inactive (17)	Fails to confirm marginal activity in 79122. Terminate testing.
05070	AD33996	79122	100	SQ	Active (21)(M)	Unconfirmed
		79157	100	SQ	Inactive (18)	Fails confirm marginal activity in PCT 79122.
05122	BH73449	79157	100	SQ	Active (23)(M)	Unconfirmed
05133	BH50099	79171	100	SQ	Active (51)	Unconfirmed
05134	BH67549	79171	100	SQ	Active (23)(M)	Unconfirmed
05136	BH73216	79171	100	SQ	Active (23)(M)	Unconfirmed

TABLE 6 (continued)

Brazil Number	Bottle Number	FY79 Test Run (Julian date)	Drug Administration		Reported Test Results (Mean No. Worms/Liver)	Confirmation
			Dosage (mg/kg)	Route		
05180	AF50488	79171	100	SQ	Active (23)(M)	Unconfirmed
05190	AT11169	79171	100	SQ	Active (28)	Unconfirmed
05191	AT13518	79171	100	SQ	Active (36)	Unconfirmed
05194	AT16897	79171	100	SQ	Active (25)	Unconfirmed
05198	AT27194	79171	100	SQ	Active (20)(M)	Unconfirmed
05199	AT27738	79171	100	SQ	Active (22)(M)	Unconfirmed
05209	AT33665	79185	100	SQ	Active (21)(M)	Unconfirmed
05210	AT33852	79185	100	SQ	Active (23)(M)	Unconfirmed
05214	AT48559	79185	100	SQ	Active (23)(M)	Unconfirmed
05229	AT70337	79185	100	SQ	Active (20)(M)	Unconfirmed
05232	AT71414	79185	100	SQ	Active (20)(M)	Unconfirmed
05622	BJ08205	79122	100	SQ	Toxic	NA

TABLE 6 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>FY79 Test Run (Julian Date)</u>	<u>Drug Administration</u>		<u>Reported Test Result (Mean No. Worms/Liver)</u>	<u>Confirmation</u>
			<u>Dosage (mg/kg)</u>	<u>Route</u>		
05622	BJ08205	79157	10	SQ	Inactive (17)	NA
			20	SQ	Active (22)(M)	Confirmed
			40	SQ	Toxic	NA

TABLE 7

Compounds screened in the Primary Curative Test (PCT) system against S. mansoni during FY79 and determined to be toxic (T) at the test dosages indicated. Repetition of the same dose indicates that the compound was retested for confirmation. The lack of a toxicity indicator (T) represents non-toxicity and inactivity at that dosage. All compounds were administered subcutaneously.

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
02873	BH07945	40 T	04743	AD02268	10 50 T 100 T
02902	BH08255	40 T	04750	AD03194	50 100 T
04582	BE19397	50 T 100 T	04756	AD03667	10 50 T 100 T
04697	BH57865	50 100 T	04757	AD03685	10 50 T 100 T
04724	AC29826	50 T 100 T	04771	AD38419	50 T 100 T
04731	AC74956	50 100 T			
04732	AC75088	10 50 T 100 T			

TABLE 7 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
04796	AB09169	50 100 100 T	04846	AC02754	50 T 100 T
04825	AB81401	10 50 T 100 T	04852	AC13604	50 100 T
04835	AB91470	50 100 T	04885	AD49761	50 100 T
04836	AB92799	10 20 50 T 100 T	04890	AC96345	100 T
04842	AB92721	50 100 T	04938	AE00170	10 20 50 T 100 T
04843	AC00090	50 100 T	04943	AE02405	10 20 50 T 100 T
04845	AC01999	50 100 T	04946	AE16294	10 20 T 50 T 100 T

TABLE 7 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
04953	AE27377	10 20 50 T 100 T	05000	AV36997	100 T
04966	AE35735	50 100 T	05001	AV37136	50 T 100 T
04972	AE48509	50 100 T	05002	AC37145	50 T 100 T
04997	AV36620	10 T 20 T 50 T 100 T	05005	AV37314	50 T 100 T
4998	AV36639	10 20 T 50 T 100 T	05007	AV37458	50 T 100 T
04999	AV36728	10 20 50 T 100 T	05014	BH65910	50 100 T
			05043	AD06113	50 100 T
			05055	AD16717	50 100 T
			05083	AD37752	100 T

TABLE 7 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
05109	BH70126	100 T	05261	AT85838	100 T
05116	BH73252	100 T	05265	AT86086	100 T
05123	BH73458	100 T	05268	AT88213	100 T
05139	BH67503	100 T	05269	BH81825	100 T
05142	BH72611	100 T	05271	AS03626	100 T
05187	AF89667	100 T			
05188	AS00465	100 T			
05192	AT13912	100 T			
05193	AT13949	100 T			
05207	AT31607	100 T			
05208	AT33521	100 T			
05228	AT70097	100 T			
05248	AT78217	100 T			

TABLE 8

Compounds screened in the Primary Curative Test (PCT) System against *S. mansoni* during FY79 and determined to be inactive and non-toxic at the test dosages indicated. All compounds were administered subcutaneously unless otherwise indicated (Gav = oral administration by gavage). A test dosage appearing twice for the same compound represents a retest at that dosage.

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
01011	BB92695	40 Gav	04795	AB07414	100
02890	BH08120	40	04797	AB09374	100
04587	AB60519	160	04798	AB09927	100
04701	BH57936	160	04799	AB09981	100
04754	AD03603	50 100	04801	AB11785	100 100
04779	AH61294	50 100	04802	AB13298	100
04788	BH58979	100	04803	AB15578	100 100
04789	BH58988	100	04805	AB16557	100
04793	AB02160	100	04806	AB18775	100
04794	AB06462	100	04808	AB31027	100

TABLE 8 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
04809	AB46064	100	04823	AB73678	100
04810	AB47061	100	04824	AB79394	100
04811	AB47187	100	04826	AB81689	100
04812	AB53390	100	04827	AB85687	100
04814	AB61267	100	04828	AB85696	100 100
04815	AB67278	100	04829	AB85749	100
04816	AB64429	100	04830	AB86309	100
04817	AB67536	100	04831	AB88633	100
04818	AB68462	100	04832	AB88697	160
04819	AB68864	100	04834	AB89265	100
04820	AB68908	100	04837	AB94551	100
04821	AB70640	100	04838	AB95496	100
04822	AB73294	100			

TABLE 8 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
04839	AB95503	100	04857	AC13917	100
04840	AB95781	100	04858	AC19311	100
04841	AB95807	100	04859	AC30703	100
04844	AC00287	100	04860	AC43317	100
04847	AC03591	100	04861	AC80098	100
04848	AC03635	100	04862	AC83786	100
04849	AC03831	100	04863	AC84596	100
04850	AC12438	100	04864	AC84612	100
04851	AC13597	100	04865	AD41630	100
04853	AC13819	100	04866	AD41658	100
04854	AC13846	100	04867	AD41809	100
04855	AC13882	100	04868	AD41818	100
04856	AC13891	100	04869	AD41925	100

TABLE 8 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
04870	AD41952	100	04883	AD42879	100
04871	AD42388	100	04884	AD42931	100
04872	AD42397	100	04886	BG10358	100
04873	AD42413	100	04887	BG10438	100
04874	AD42422	100	04888	BG12147	100
04875	AD42431	100	04889	BH30784	100
04876	AD42459	100	04891	AD43901	100
04877	AD42468	100	04892	AD43992	100
04878	AD42486	100	04893	AD44006	100
04879	AD42673	100	04894	AD44015	100
04880	AD42682	100	04895	AD44033	100
04881	AD42708	100	04896	AD44042	100
04882	AD42842	100	04897	AD44051	100

TABLE 8 (continue)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
04898	AD44542	100	04913	AD52302	100
04901	AD45969	100	04914	AD53523	100
04902	AD45996	100	04915	AD53890	100
04903	AD47927	100	04916	AD70391	100
04904	AD48184	100	04917	AD71647	100
04905	AD48193	100	04918	AD72377	100
04906	AD48504	100	04919	AD73025	100
04907	AD48577	100	04920	AD73721	100
04908	AD48693	100	04921	AD74808	100
04909	AD48700	100	04922	AD76508	100
04910	AD49716	100	04923	AD77544	100
04911	AD49725	100	04924	AD77185	100
04912	AD50728	100	04925	AD77201	100

TABLE 8 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
04926	AD77416	100	04941	AE00732	100
04927	AD77649	100	04942	AE02370	100
04928	AD77998	100	04944	AE03019	100
04929	AD78217	100	04945	AE03064	100
04930	AD84500	100	04946	AE03895	100
04931	AD85945	100	04947	AE04052	100
04932	AD86317	100	04948	AE07204	100
04933	AD86488	100	04949	AE07213	100
04934	AD86899	100	04950	AE07222	100
04935	AD87663	100	04951	AE07231	100
04936	AD87770	100	04952	AE12394	100
04937	AD87921	100	04953	AE12456	100
04940	AE00714	100	04959	AE27411	100

TABLE 8 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
04960	AE28310	100	04976	AE49300	100
04961	AE29657	100	04977	AE49328	100
04962	AE30169	100	04978	AE53055	100
04963	AE31095	100	04979	AE56010	100
04964	AE35717	100	04980	AE56216	100
04965	AE35726	100	04981	AE56225	100
04967	AE38450	160	04982	AE56252	100
04968	AE38656	100	04983	AE56270	100
04969	AE43068	100	04984	AE58363	100
04971	AE48483	100	04985	AE59404	100
04973	AE49239	160	04986	AE73717	100
04974	AE49266	100	04987	AE75597	100
04975	AE49275	100	04988	AE83400	100

TABLE 8 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
04989	AE84425	100	05009	AV55778	100
04990	AE86670	100 200	05010	AX52311	100
04991	AE86689	100	05011	AX52704	100
04992	AE96238	100	05012	BH65778	100
04993	AE14482	100	05013	BH65858	100
04994	AF52491	100	05015	BH65947	100
04995	AF55410	100	05016	BH65956	100
04996	AT14071	100	05017	BH65965	100
05003	AV37154	100	05018	BH65974	100
05004	AV37216	100	05019	BH66346	100
05006	AV37323	100	05020	BH66364	100
05008	AV38026	100	05021	BH66408	100
			05022	BH66435	100

TABLE 8 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
05023	BH66444	100	05036	BH70162	100
05024	BH66480	100	05037	BH70171	100
05025	BH66499	100	05038	BH70180	100
05026	BH66631	100	05039	BH70199	100
05027	BH66757	100	05040	AD04468	100
05028	BH66819	100	05041	AD04511	100
05029	BH66828	100	05042	AD05661	100
05030	BH66873	100	05045	AD08457	100
05031	BH72353	100	05046	AD08466	100
05032	AF35963	100	05047	AD08939	100
05033	BH69945	100	05048	AD10359	100
05034	BH69990	100	05049	AD10420	100
05035	BH70144	100	05050	AD10528	100

TABLE 8 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
05051	AD10733	100	05065	AD28780	100
05052	AD10751	100	05066	AD29698	100
05053	AD10868	100	05067	AD29705	100
05054	AD13627	100	05069	AD32784	100
05056	AD18088	100	05071	AD34859	100
05057	AD18882	100	05072	AD35589	100
05058	AD20319	100	05073	AD36380	100
05059	AD21718	100	05074	AD36424	100
05060	AD23472	100	05075	AD36433	100
05061	AD26848	100	05076	AD36577	100
05062	AD28011	100	05077	AD36595	100
05063	AD28182	100	05078	AD36675	100
05064	AD28655	100	05079	AD36773	100

TABLE 8 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
05080	AD36906	100	05094	AD41694	100
05081	AD36915	100	05095	AD41854	100
05082	AD36960	100	05096	AD41907	100
05084	AD37921	100	05097	AD41970	100
05085	AD38277	100	05098	AD45227	100
05086	AD38795	100	05099	AD46779	100
05087	AD39176	100	05100	AD46966	100
05088	AD39578	100	05101	AD48326	100
05089	AD39667	100	05105	BH66551	100
05090	AD41185	100	05106	BH58951	100
05091	AD41274	100	05107	BH58997	100
05092	AD41283	100	05108	BH70108	100
05093	AD41569	100	05110	BH72915	100

TABLE 8 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
05111	BH72942	100	05128	BH73823	100
05112	BH72951	100	05130	BH16980	100
05113	BH72960	100	05132	BH50071	100
05114	BH72997	100	05135	BH67558	100
05115	BH73243	100	05140	BH72488	100
05117	BH65250	100	05141	BH72497	100
05118	BH67496	100	05143	BH72666	100
05120	BH73421	100	05144	BH72817	100
05121	BH73430	100	05151	BH76253	100
05124	BH73467	100	05152	BH76262	100
05125	BH73494	100	05181	AF61589	100
05126	BH73501	100	05182	AF70355	100
05127	BH73510	100	05183	AF82328	100

TABLE 8 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
05184	AF86988	100	05206	AT31330	100
05185	AF88044	100	05211	AT34055	100
05186	AF86768	100	05212	AT34199	100
05189	AT10822	100	05213	AT48282	100
05195	AT18275	100	05215	AT48675	100
05196	AT25985	100	05216	AT48899	100
05197	AT26428	100	05217	AT49065	100
05200	AT28093	100	05218	AT57389	100
05201	AT28182	100	05219	AT58162	100
05202	AT28253	100	05220	AT63323	100
05203	AT28280	100	05221	AT63734	100
05204	AT30913	100	05222	AT64151	100
05205	AT30931	100	05223	AT64428	100

TABLE 8 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
05224	AT64704	100	05241	AT76802	100
05225	AT65130	100	05242	AT76893	100
05226	AT65596	100	05243	AT77210	100
05227	AT65658	100	05244	AT77283	100
05230	AT70542	100	05246	AT77729	100
05231	AT71325	100	05249	AT78511	100
05233	AT71781	100	05250	AT78708	100
05234	AT75056	100	05251	AT78940	100
05235	AT75298	100	05253	AT79367	100
05237	AT75458	100	05254	AT79590	100
05238	AT76615	100	05255	AT79670	100
05239	AT76624	100	05256	AT81027	100
05240	AT76795	100	05257	AT83183	100

TABLE 8 (continued)

<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>	<u>Brazil Number</u>	<u>Bottle Number</u>	<u>Dosage (mg/kg)</u>
05258	AT83272	100	05276	AS29068	100
05259	AT84840	100	05277	AS34587	100
05260	AT85829	100	05347	BH86464	40
05262	AT85981	100	05348	BH86473	40
05263	AT86022	100	05371	BH89536	40
05264	AT86040	100	05591	AT19058	100
05266	AT86219	100			
05267	AT86728	100			
05270	AS01524	100			
05272	AS06814	100			
05273	AS08541	100			
05274	AS10649	100			
05275	AS11851	100			

PART II. CLINICAL, EPIDEMIOLOGICAL, IMMUNOLOGICAL AND ENTOMOLOGICAL STUDIES ON MALARIA IN AMAZONAS, BRAZIL, ALONG THE ITUXI RIVER.

1. General:

A program of studies on malaria in the Amazon Basin was initiated in CY 1978. The field site for these studies is along the Ituxi River, southwest of Labrea, Amazonas, Brazil (Figure 1). The operational headquarters and laboratories in the Núcleo de Medicina Tropical e Nutrição (Center of Tropical Medicine and Nutrition) at the University of Brasília provide the logistical and technical support for our studies in the field. Over-all program objectives are to evaluate: a) the clinical and epidemiological aspects of malaria, b) the immunological aspects of malaria with increased emphasis on *in vitro* drug susceptibility testing, and c) the ecology and population dynamics of malaria vectors. The information from these, and associated, studies is fundamental to an understanding of the mechanisms affecting continued malaria transmission despite ongoing control measures in these areas.

2. Description of the Field Study Area:

The Ituxi River region is an excellent area for conducting studies on malaria ecology due to the existence of a high level of disease transmission in spite of active control efforts. The Ituxi is a branch river of the larger Purus River. The Ituxi-Purus confluence is located approximately 10 kilometers west of Labrea in Amazonas State. The Ituxi headwaters are found in the states of Acre and Amazonas. The terra firme and igapo habitats are the most frequently encountered habitats along the river (terra firme is highland that is never inundated by the river; igapo is composed of low areas that are inundated for several months each year). The Ituxi River residents comprise a widely distributed and stable community. They generally have been born and raised on this river system. Houses normally are built on terra firme and families earn their livelihood by collecting rocks for construction, rubber, latex and castanhas do Pará. Subsistence farming, hunting, and fishing are other principal activities.

3. Clinical and Epidemiological Studies of Malaria in Amazonas, Brazil:

a. Introduction: Although measures to eradicate malaria are applied throughout the state of Amazonas, the incidence and prevalence of this disease remains high. Malaria surveillance data obtained from Su-

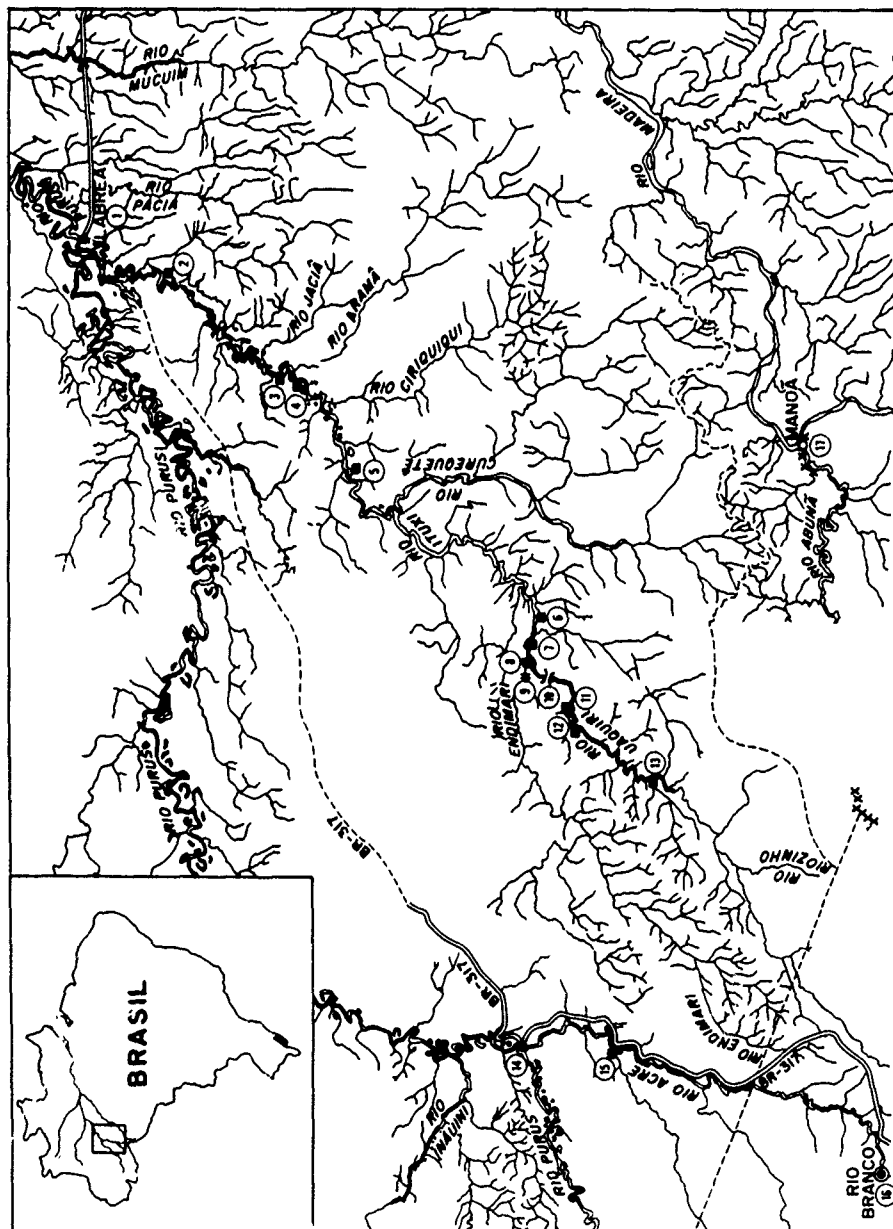


Fig 1

CIDADES E VILAS

- | | | |
|------------|--------------------------------|--------------------|
| ① LABREA | ⑦ SANÚACA | ⑬ BOCA DO RIOZINHO |
| ② MISSÃO | ⑧ FORTALEZA DO ITUXI | ⑭ BOCADO ACRE |
| ③ PALMAPI | ⑨ CACHOEIRA DO MEIO (DEPÓSITO) | ⑮ FLORIANO PEIXOTO |
| ④ FLORESTA | ⑩ CACHOEIRA DA ÁGUA PRETA | ⑯ RIO BRANCO |
| ⑤ CAMARGO | ⑪ MATIPUÃ | ⑰ MANOÃ |
| ⑥ BACABAL | ⑫ BATATA | |

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perintendencia de Campanhas de Saúde Pública (SUCAM) reveal that Amazonas remains in the group of states within Brazil where malaria control is yet to be achieved. The slide positivity rates for these states exceeded those from the remainder of Brazil by approximately twenty-fold for each of the years 1974 through 1978 (Table 9). The average annual slide positivity rate for the municipality of Labrea, Amazonas, was 11.28% during this same period, although it appears that the disease may be receding somewhat in the urban areas (Table 10). These data evidence the continued significance of malaria in Brazil and serve as indicators of the amount of work yet to be done toward the eventual goal of eradication.

b. Objectives: The objectives of the clinical and epidemiological studies are to:

1) determine the influence of migration on the perpetuation of malaria endemicity;

2) establish the clinical events of malaria infections for future comparisons and evaluations;

3) determine the level of antimalarial antibodies in this population and correlate this with spleen sizes;

4) monitor boat traffic on the river to determine the impact of population movement on malaria endemicity.

c. Methods: Methods used to accomplish these objectives are:

1) accurate mapping of the study area and censusing to determine the number and location of residents along the river;

2) the initial clinical examination of at least 85-90% of the population with special interest in slide positivity rates and spleen size;

3) the collection of sera for antimalarial serological testing;

4) the performance of follow-up examinations and repeated testing.

d. Progress: The preliminary mapping and the census of the study area have been accomplished. Although the exact percentage of persons examined, as well as certain other clinical correlations, is unavailable due to incomplete computer analysis, it appears that the projected 85-90% of the population has been examined. The total study population consists of 155 families including 941 persons, or an average of 6.07 persons/family. A total of 1153

TABLE 9

Distribution of the index of malaria incidence in Brazil by slide positivity, 1974-1978, for all species of malaria (Source: SUCAM).

Geographical area of Brazil according to degree of malaria eradication	Percent Slide Positivity Rate				
	1974	1975	1976	1977	1978
Eradication nearing completion.....	0.7	0.7	0.5	0.5	0.5
Eradication only on a long-term basis..	9.8	10.1	10.5	10.5	10.9

TABLE 10

Malaria slide positivity rates for the municipality of Labrea, Amazonas, 1974-1978 for all species of malaria (Source: SUCAM).

Year	Number of Slides Examined	Number of Positives	Percent Positive
1974	2264	208	9.18
1975	1205	165	13.69
1976	1774	305	17.19
1977	856	73	8.52
1978	1841	145	7.87
Totals	7940	896	11.28

slides have been examined with a total of 90 positives (7.8%), 43 being Plasmodium falciparum and 47 being P. vivax. The age distribution of these persons with positive smears by species of malaria is given in Table 11.

A total of 1014 serum and filter paper samples have been tested from this population, although filter paper data gave sufficiently poor results to not be included in the analysis. Of all sera tested, 91.64% were positive for antimalarial IgG and 30.54% were positive for antimalaria IgM. The age distribution of persons with positive serologies is shown in Table 12. The low incidence of malaria antibodies, notably IgM, in the younger age groups is of interest because of the high rate of slide positivity in these same groups. One possible explanation that has been considered is that prompt chloroquine treatment of these younger individuals due to a more severe clinical course and earlier appearance of fever may result in decreased antibody production. The ubiquitous availability of chloroquine and the presumptive treatment of all fevers with this drug lend some credibility to this theory, but it is far from proven at this point and further investigation is needed.

Another interesting correlation exists between spleen rates and the serological data. Of 217 persons with a palpable spleen, 197 (90.78%) had significant levels of IgG and the remaining 20 persons without positive serologies were all less than 20 years of age. In the remaining group of 112 persons without a palpable spleen, 101 (90.18%) had positive IgG serologies and the 11 persons with negative serologies were again all under 20 years of age. These data indicate that serological screening for the presence of antimalarial IgG is a better measure of malaria prevalence than spleen surveys, certainly in persons over 20 years of age. It should also be noted that there was a much weaker correlation between spleen rates and the presence of antimalarial IgM. Further study of these relationships is desirable.

Table 13 presents comparison data between a group of 63 persons who had temporarily migrated into the interior of the jungle and 63 persons permanently residing on the river. Based on antimalarial IgM rates, the population remaining near the river appear to have a higher incidence of disease than the migrants, implicating areas nearer the river as being more active sites of transmission. Antimalarial IgG rates were higher in the migrant population, although not markedly so, and say little regarding the source of their previous exposure, the interior areas versus the riverine areas.

TABLE 11

Age distribution of malaria slide positivity in the Ituxi River population by species of malaria.

<u>Age Group</u>	<u>Number of Positive Slides</u>	
	<u>Plasmodium falciparum</u>	<u>Plasmodium vivax</u>
0-4	15	20
5-9	10	15
10-14	12	6
15-19	4	1
20-24	0	1
25-29	1	2
30-34	1	2
41-49	0	0
≥ 50	0	0
Totals	43	47

TABLE 12

Percent positivity of antimalarial serologies of the Ituxi River population by age.

<u>Age Group</u>	<u>Percent Postive Serologies</u>	
	<u>IgG</u>	<u>IgM</u>
0-4	81	0
5-9	67	4
10-14	87	18
15-19	92	21
20-24	100	36
25-29	100	53
30-40	97	47
41-49	100	33
≥ 50	95	54

TABLE 13

Comparison of antimalarial seropositivity between a group of migrants to the jungle interior and a non-migrant riverine population, Ituxi River study area.

<u>Population</u>	<u>Percent Serological Positives</u>	
	<u>IgG</u>	<u>IgM</u>
Migrants	92.4	26.4
Non-migrants	86.8	37.7

In another attempt to clarify the transmission patterns of malaria within this area, a group of 42 persons who travel the river by boat, but who do not live in the study region, were studied serologically. Of these, a total of 27 (64.28%) were positive for IgG and 13 (30.95%) were positive for IgM. Since the rate of positive serologies in this group, particularly for IgM, does not differ markedly from that of the resident population, the impact that these river travelers have on the overall transmission patterns of malaria is questionable. However, these persons may play an important role in the annual reintroduction of *P. falciparum* into the study area after the apparently temporary disappearance of this parasite during the dry season of certain years. This contrasts with *P. vivax* which occurs at reduced, but still significant, levels throughout the dry season, mainly in the form of recrudescence disease. The mobile populations also may be effective carriers of malaria between family units during the malaria transmission season.

Further analyses of presently available data are continuing and more field studies in the Ituxi region are being planned for FY80.

4. Immunological studies of malaria in Amazonas, Brazil:

a. Introduction: The program in malaria immunology was established at the University of Brasilia in the Center of Tropical Medicine and Nutrition in October, 1978. Suitable laboratory space was selected, necessary physical modifications were made, and equipment was installed during a start-up phase of approximately four and one-half months. Since that time, much progress has been made toward the

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initial goal of providing full laboratory support for the clinical and epidemiological studies of malaria presently being conducted from this center. Two technicians have been fully trained in all of the techniques utilized in the routine operation of the laboratory and efficiently assist in all aspects of the ongoing research. The malaria serological studies and the necessary support activities of this operation are functioning at a level of proficiency to allow expanded efforts in the areas of drug susceptibility testing and the study of the in vitro cultivation characteristics of local strains of P. falciparum as they are obtained from the various study areas.

b. Objectives: The objectives of the malaria immunology program are to perform:

- 1) malaria serological testing;
- 2) drug susceptibility testing of Brazilian strains of P. falciparum.
- 3) collection, cryopreservation, and storage in stabulate form of strains of P. falciparum to provide material for ongoing studies and to serve as a reference in monitoring future patterns of drug susceptibility in the Amazon region of Brazil;
- 4) laboratory support of ongoing studies of patients with tropical splenomegaly syndrome from the Ituxi study region; and
- 5) other tests, such as the species-specific indirect fluorescent antibody test, to study the immunologic characteristics of malaria in Brazil as logistical capability permits.

c. Methods: Methods used to accomplish these objectives are:

- 1) maintenance of a constant and dependable source of malaria antigen by the on-site in vitro cultivation of P. falciparum (2,3) using blood components locally available from the teaching hospital in Sobradinho, DF, Brazil;
- 2) routine use of the indirect fluorescent antibody test (IFAT) as the standard serologic test for determining levels of antimalarial antibodies (4) using commercial anti-IgG and anti-IgM fluorescein-labelled globulins;
- 3) use of the in vitro technique of chloroquine susceptibility testing (5) to study the drug resistance patterns of local strains of P. falciparum.
- 4) development of the species-specific malaria IFAT (6) using locally obtained P. vivax antigen from patients infected with this organism as logistical capabilities permit; and

5) quantitative determination of IgM levels, particularly in patients with tropical splenomegaly syndrome, by the radial immunodiffusion assay system (7) using commercially-acquired kits.

d. Progress: In February, 1979, the in vitro cultivation of P. falciparum (Strain Cbl, Department of Immunology, Walter Reed Army Institute of Research) was initiated using the tissue culture flask/mixed gas system. This strain has demonstrated excellent growth characteristics and, until recently, has served as the standard laboratory strain for antigen production. The cultivation has been interrupted voluntarily on various occasions by cryopreservation of the stock material and then restarted by deglycerinization as the need for antigen to prepare slides for the IFAT has dictated. The candle jar culture system using either standard Petri dishes or 96-well microtiter plates has been incorporated with excellent technical results, and in many ways is preferred over the flask system because of its greater simplicity, economy, and the facility of medium changes.

On 25 July 1979, at a field site on the Ituxi River, one strain of P. falciparum from an untreated patient was cryopreserved. A total of 7 NUNC tubes of stablate was prepared in the field and 2 of these were used to inoculate 4 flasks of medium 1640-HEPES/10% fresh frozen plasma and fresh, washed erythrocytes on 4 September 1979. (Unforeseen problems encountered in scheduling air transportation of the liquid nitrogen cannister back to Brasilia account for the delay between time of collection of this strain and subsequent cultivation attempts). Active parasite growth occurred in these initial cultures and the strain continues to thrive in continuous cultivation. It has presently been maintained for more than one month in this system. High parasitemias are obtained easily and two lots of antigen slides have already been produced, as well as additional organisms for cryopreservation. It has also been placed into the candle jar system and studies of its in vitro growth characteristics are in progress.

Initial observations indicate that this strain exhibits a rate of growth in culture similar to or slightly higher than strain Cbl. The new strain has been given the name "Ituxi 084", after the location in which it was collected and the computer card number of the patient. The cultivation of this strain provides a preliminary indication that many such strains may be adapted to the continuous culture system to allow in depth investigation of their drug susceptibility patterns. This strain is presently being studied in the in vitro chloroquine susceptibility test system and useful data will be available in the near future pertaining to its pattern of drug response.

The technical capability to perform in vitro drug susceptibility testing in the field presently exists. On the field trip earlier

in CY 1979, virtual cessation of P. falciparum transmission due to an unseasonably severe dry season precluded the large scale implementation of this test, however. Additional field trips later in CY 79 to a study area near Manaus and early in CY 1980 to Ituxi are planned with the expressed objectives of performing field drug testing and collecting additional strains for laboratory cultivation and study. These studies should provide much objective data regarding the current prevalence of chloroquine-resistant P. falciparum in several fairly representative areas of the Brazilian Amazon basin.

Since March, 1979, the IFAT has been in full, routine operation to support the ongoing epidemiological studies of malaria from this center. Depending on the number of designated readers, 48 or 96 tests have been performed on a daily basis since the initial standardization of this procedure. Excellent technical results are being obtained from the commercially-acquired antiglobulins and the P. falciparum antigen cultivated here in the laboratory.

In addition to the routine serological determinations, the preliminary results of which were presented above, a study was conducted to evaluate the applicability of the filter paper method of collecting blood for subsequent serological testing. The study material consisted of one group of sera and two groups of filter paper specimens obtained simultaneously from the same patients. The sera and one group of filter papers were stored at - 20°C from the time of collection to time of testing, while the second group of filter papers was stored at room temperature for this same period. Approximately two months elapsed between time of collection and subsequent testing in the IFAT system. The resultant data (Table 14) indicate that neither group of filter paper specimens compared favorably to the serum samples in demonstrating the presence of antimalarial antibody, either IgG or IgM. These data, in addition to similar findings obtained from other filter paper specimens from the Ituxi population, indicate that this method of sample collection is almost certainly producing many false negative results and will not be used on a routine basis in further serological studies of malaria on the Ituxi River.

Preliminary studies are proceeding with the radial immunodiffusion assay of IgM antibody levels in a small group of tropical splenomegaly patients from the Ituxi study area. Initial data (Table 15) indicate that high levels of circulating IgM are present in these patients. The titers of malarial antibodies, also presented in Table 15, confirm that at least a portion of this circulating IgM is specific for malaria, although as has been earlier observed in this syndrome (8), other types of IgM appear to occur in significant quantities in these patients. Further investigation of these relationships is proceeding.

TABLE 14

Malarial antibody positivity rates in a controlled study of serum and filter paper specimens simultaneously collected from the same individuals, Ituxi River study area.

Sample	Percent Positive	
	IgG	IgM
Serum (-20°C storage)	100	25.5
Filter papers (-20°C storage)	72.3	6.3
Filter papers (Ambient temperature storage)	27.6	0

TABLE 15

Antimalarial IgG and IgM titers by the immunofluorescent antibody test and total circulating IgM levels by radial immunodiffusion assay in four tropical splenomegaly patients, Ituxi River study population.

Patient	Malarial antibody titers		Level of total circulating IgM (mg/dl)
	IgG	IgM	
M. S. O.	1:1280	1:80	460
M. V. P.	1:320	1:80	665
F. S. O.	1:1280	1:320	2300
C. S. O.	1:5120	1:1280	2300

5. Entomological Studies on Malaria in Amazonas, Brazil.

a. Introduction: The Amazon Basin is classified as "refractory" to malaria control efforts. This classification is based on the persistence of malaria transmission in spite of the control program. The success of the program rests on controlling the vector populations by house spraying with DDT. Obviously, "refractoriness" indicates that due to some condition or complex of conditions the treatment of houses with DDT does not interdict malaria transmission as expected. As part of an integrated approach to define the causative factors for continued malaria transmission, emphasis in the vector studies has been to establish the broad parameters of vector behavior that have direct impact on the effectiveness of house spraying with DDT. Our field work along the Ituxi River brought us in contact with another entomological phenomenon that might influence the effectiveness of malaria vector control efforts. This occurs in the form of a male bee that seems to actively remove DDT from treated houses. Preliminary observations have been made on these bees; thus, the results of our entomological investigations will be presented in two separate categories as 1) the ecology and populations dynamics of malaria vectors and 2) the role of euglossine bees in the removal of DDT from sprayed houses.

b. The Ecology and Population Dynamics of Malaria Vectors.

1) Objective: To describe the behavioral, morphological and physiological characteristics of the malaria vectors in Brazil, with special emphasis on Anopheles darlingi Root.

2) Background:

The definitive research efforts on malaria in the Amazon region were conducted from 1930-1950 (9, 10, 11, 12, 13). Results from these studies revealed the principal vector in the Amazon interior to be Anopheles darlingi Root. Secondary vectors were found to be An. (Nyssorhynchus) albitarsis Lynch Arribalzaga and perhaps An. (Ny.) brasiliensis (Chagas). The major vector is generally considered to be a riverine mosquito and most studies in the epidemiology of malaria have been conducted in riverine semi-urban habitats.

Anopheles darlingi is the most important vector of malaria in South America (14, 15). Because this species prefers sunlight, its greatest density is along major river valleys, and it proliferates wherever human activities result in the removal of shade-producing forest. An. darlingi is also strongly attracted to man and rests indoors (14, 16). It seems that An. darlingi are still physiologically susceptible to DDT and only recently has behavioral resistance to

DDT been reported (17). Unfortunately, no supportive data have been presented to quantify that observation.

In forest areas away from rivers, other species may transmit malaria secondarily (18). These species are shade tolerant and commonly bite and rest outdoors (14). Control by the application of residual insecticides to the interior walls of dwellings is therefore of limited value. Species which have been incriminated as secondary vectors in northeastern South America include Anopheles nuneztovari, An. triannulatus, An. oswaldoi, An. brasiliensis, An. mediopunctatus, An. albitarsis, An. bellator, An. cruzi, and An. homunculus (14, 16). However, the exact role of secondary vectors in the maintenance of malaria has not been clarified.

Anopheles nuneztovari is probably a complex comprising at least two species in northern Brazil, with some of the species being malaria vectors, others not (19, 20). There are undoubtedly other groups of sibling species among anopheline species in Brazil that are malaria vectors, e.g., An. oswaldoi. In many instances, resolution of the morphological forms and geographical strains of anophelines transmitting malaria can be accomplished only after the study of a series of individually reared specimens from many geographic areas, and in some cases, only after the comparative study of chromosome morphology (15).

3) Methods:

An entomological survey of the peridomiliary environments along the Ituxi River was conducted in July and August 1979 (Fig. 1). The survey consisted of conducting human bait collection near or in houses at sunset and of opportunistically dipping for larvae in various types of water. In the latter part of this trip a sequence of 3 all night biting collections were conducted at Floresta (Fig. 1). Collections were conducted for 30 min each hour with one team of collectors (2 men per team) in a house open on three sides while another team collected in an open area about 20 m from the house. Each hour the teams were rotated between sites and the third night the team members were changed. In addition, 2 tests for physiological resistance of An. darlingi to DDT were conducted. Test specimens were wild caught females from human bait collections. Prior to setting up each test the females were observed for 3-4 hours to identify and remove any damaged specimens. Females were not given sugar water; but were furnished with pads soaked in plain water following a 1 hr exposure to DDT treated papers. The World Health Organization test kit and test procedures were employed to conduct these tests (21).

Results from the above studies emphasized the need for an experimental house for more detailed studies on the behavior of darlingi populations. House construction at Floresta was initiated in October 1978 and completed in January 1979. The house was constructed with 1) a palm thatch roof, 2) walls constructed of palm slats, and 3) one small room with a wood plank floor and another of palm slats. The wood plank floor provided the necessary stability for work with a microscope, etc. All windows (8 in total) were of equal size so entrance and exit traps would be interchangeable. The house was wired for electricity provided by a 3 KVA generator.

The first series of detailed studies was conducted in February - March 1979, and follow-up observations were made in May - June 1979. The following study methods were employed:

a) Paired, outdoor-indoor human bait collections were conducted in a uniform manner throughout the night and day to determine the indoor-outdoor patterns of biting activity. Collections were conducted 15 min/hr by one person at each site. Collectors were continually rotated between collecting sites and teams were switched every 6 hours. Furthermore, teams were rotated between shifts every night. The all night collections were conducted simultaneously with the entrance-exit trap collections. Two series of collections throughout the day were conducted. Data will be reported from series conducted 24 - 27 February 1979 and 31 May - 7 June 1979. Temperature and humidity, was recorded every 6 hours for the first series and at hourly intervals for the May-June investigations.

b) Entrance and exit traps placed in windows were collected at 2 hr intervals throughout the night (1800-2000, 2000-2200, etc). Each trap had a sleeved opening for removing captured specimens with a mechanical aspirator. A Safari Fluorescent lamp was used to illuminate the trap interior after the entry portal to the trap was closed with towels. We began closing the traps after we observed blood engorged ♀♀ darlingi actively entering traps in response to the bright fluorescent light, i.e., they demonstrated a positive phototaxis. All specimens were identified and individuals from exit traps were examined for age grading of blood meals according to Sella's scheme for stages of blood meal digestion and ovarian development (22).

c) Sella's method for evaluating the stages of blood meal digestion and ovarian development was employed with specimens caught in exit traps to determine elapsed time after engorgement. A study was conducted in February 1979 to evaluate Sella's criteria with An. darlingi at in-house temperatures. This study was conducted by holding individual engorged females for variable periods of time; they were then killed and inspected for concordance with one of the

stages proposed by Sella. A total of 100 females, engorged on human blood, were included in this study.

d) Blood-engorged An. darlingi were collected in the peridomiciliary environment during the early evening, marked with USR fluorescent pigment 1953 and released in the house at 2200. Marking was accomplished by blowing the powder into a small holding cage containing the specimens. Periodic observations with a Black-Ray, ULV.56, long wave ultra-violet lamp were made following release to determine the preferred resting site of blood-fed specimens. These studies were conducted on 2 separate occasions (22 and 28 February 1979) and 100 specimens were marked for each study.

e) Two tests were conducted to determine the preferred resting sites of unfed specimens and the time of feeding during the night. Specimens were collected from the entrance traps, during the 1800-2000 hour interval, marked and then released within the house at 2040 hr. All specimens caught in the subsequent hourly human bait collections were inspected for the presence of marked specimens.

f) A series of resting collections were conducted 28 February-1 March 1979. Three separate collections were conducted for 5 minutes simultaneously inside the house, from the external walls and from vegetation near the house. Two series were conducted in the evening at half hour intervals from 1830 to 2105 hr. and one series in the early morning from 0540-0715. Resting adults were captured with a mechanical aspirator.

g) Studies were undertaken on the distribution of host-seeking An. darlingi populations by distance from the peridomiciliary environments. A single collector was stationed at each of 3 sites: one > 10 m from the house, another at 20 m and the 3rd at 40 m from the house. Collections were conducted simultaneously for 15 min each from 1750 to 2005 on 3 and 4 June 1979.

4) Progress:

Anopheles darlingi were consistently present in the peridomiciliary habitats along the Ituxi and Uaquire River systems. This species was also found along the lower reaches of the Endimari River. The entire River network is sparsely populated with single family dwellings that commonly have associated populations of An. darlingi.

Data from the insecticide resistance tests are presented in Table 16. The LC_{50} , estimated on log probit graph paper, with combined data from 2 tests, is 0.72% DDT and clearly within the susceptible range.

TABLE 16

Result from 2 tests for physiological resistance to DDT of ♀♀
Anopheles darlingi Root. Females were caught in human bait collec-
 tions from 1830-2030 hr at Floresta, Ituxi River, Amazonas, Brazil.
 Tests were initiated at 2300 hr on the night of collecting the test
 specimens.

<u>Test No.</u>	<u>% DDT</u>	<u>Number tested</u>	<u>Number moribund or dead</u>
1*	0.0	40	5
	0.5	40	17
	1.0	36	28
	2.0	39	36
	4.0	38	38
2**	0.0	30	4
	0.5	39	22
	1.0	24	12
	2.0	42	39
	4.0	41	41

*

Test performed 13-14 July 1978.

**

Test conducted 1-2 August 1978.

A bimodal pattern of biting activity was documented for An. darlingi both inside and outside of a non-enclosed house at Floresta in August 1978. Peak activity was during, and immediately after, sunset and at sunrise (Fig. 2). It is significant that no marked differences were found in the activity cycles of darlingi in the house and in an open area near the house.

Two separate series of studies on activity patterns in a house with complete walls were conducted. Uniform methods were applied during both; thus, findings are presented as combined results with reference to the separate series as study 1 (February - March 1979) and study 2 (May - June 1979). Weather conditions were different for studies 1 and 2 with temperature range limits of 24-31°C recorded for study 1 and 16-30°C for study 2.

A bimodal pattern of biting activity in the peridomiciliary environment (within 10 m of the house) was documented in the August 1978 series of human bait collections and in studies 1 and 2 in 1979 (Figs. 2, 3 and 4). Peak activity occurred during, and preceding, sunset with a secondary peak at sunrise (at approximately 0600). The secondary peak was not well expressed in the study 2 collections. These activity patterns were compared by calculating cumulative per cent distributions for each and testing in the Kolmogorov-Smirnov two sample test (23). No significant differences were detected in these analyses.

Human bait collections were conducted inside the experimental house, during studies 1 and 2, to determine the pattern of activity within a completely enclosed house. Collections in study 1 revealed a sharp increase in activity after sunset with more or less continuous activity throughout the night. There was no detectable peak in activity at sunset or sunrise. The minimum temperature recorded during these collections was 24°C.

In-house biting activity during study 2 was most intense at, and 3 h following, sunset. After 2147 the activity dropped and remained low the rest of the night. A comparison of results from studies 1 and 2 with the Kolmogorov-Smirnov two sample test revealed significant difference ($p < 0.01$) between the 2 activity patterns.

It is reasonable to explain deviations from expected activity patterns by notable differences in study conditions. Therefore, we hypothesized that low temperatures suppressed host-seeking activity of An. darlingi during study 2. We tested this hypothesis by analyzing sequential collections for 2 activity intervals with the Kendall Rank Correlations and Kendall Partial Rank Correlation Coefficients (23). Data available for analysis consisted of numbers collected per collection, time of collection and temperature at the time of

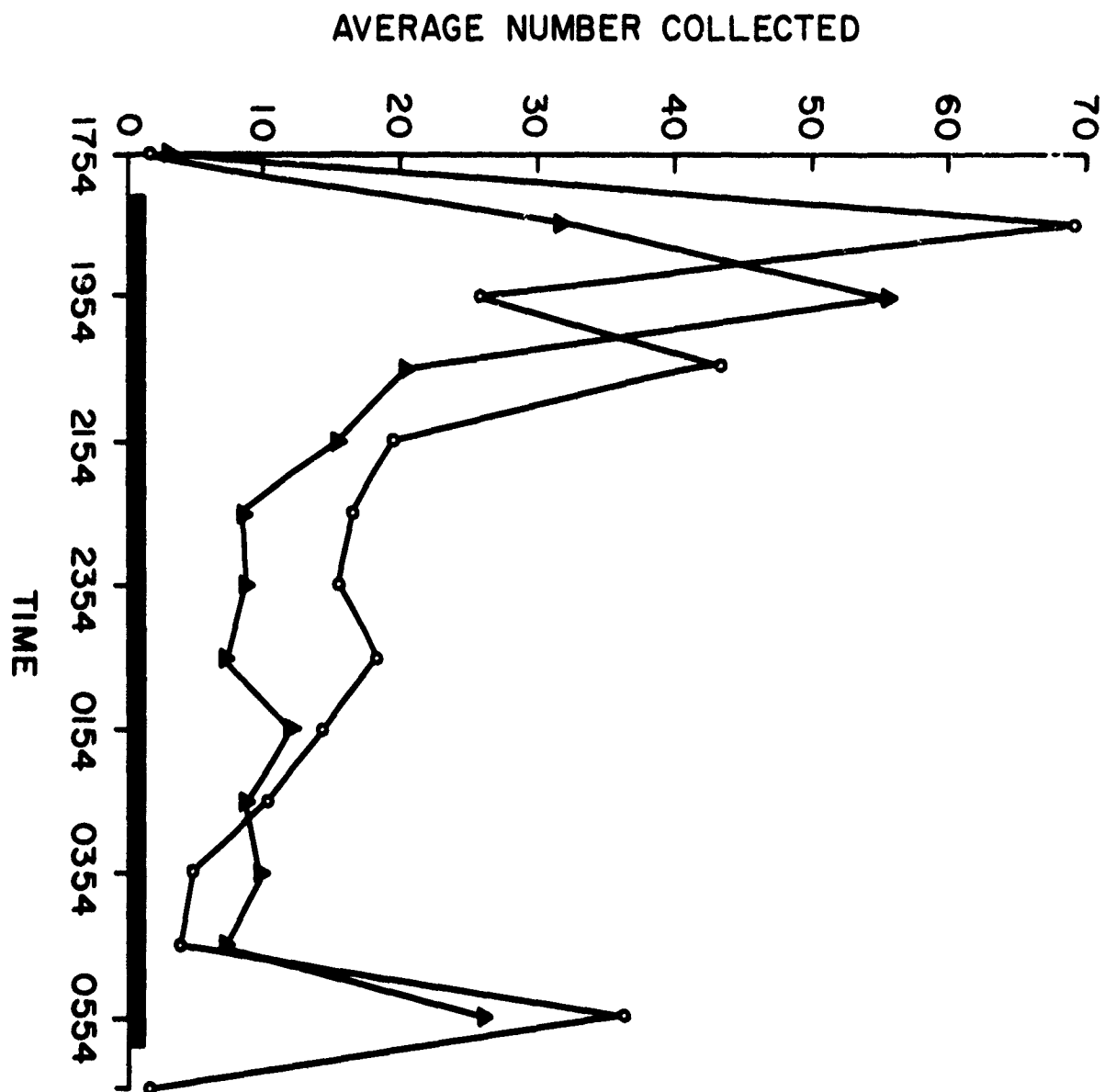


Figure 2. Numbers of *Anopheles darlingi* Root from 3 nights of human bait collections at Floresta, Ituxi River, Amazonas, Brazil in August 1978. Collections conducted by 2 collectors for 30 min. each hour (○—○ inside of a house with one wall only; ▲—▲ open area near the house).

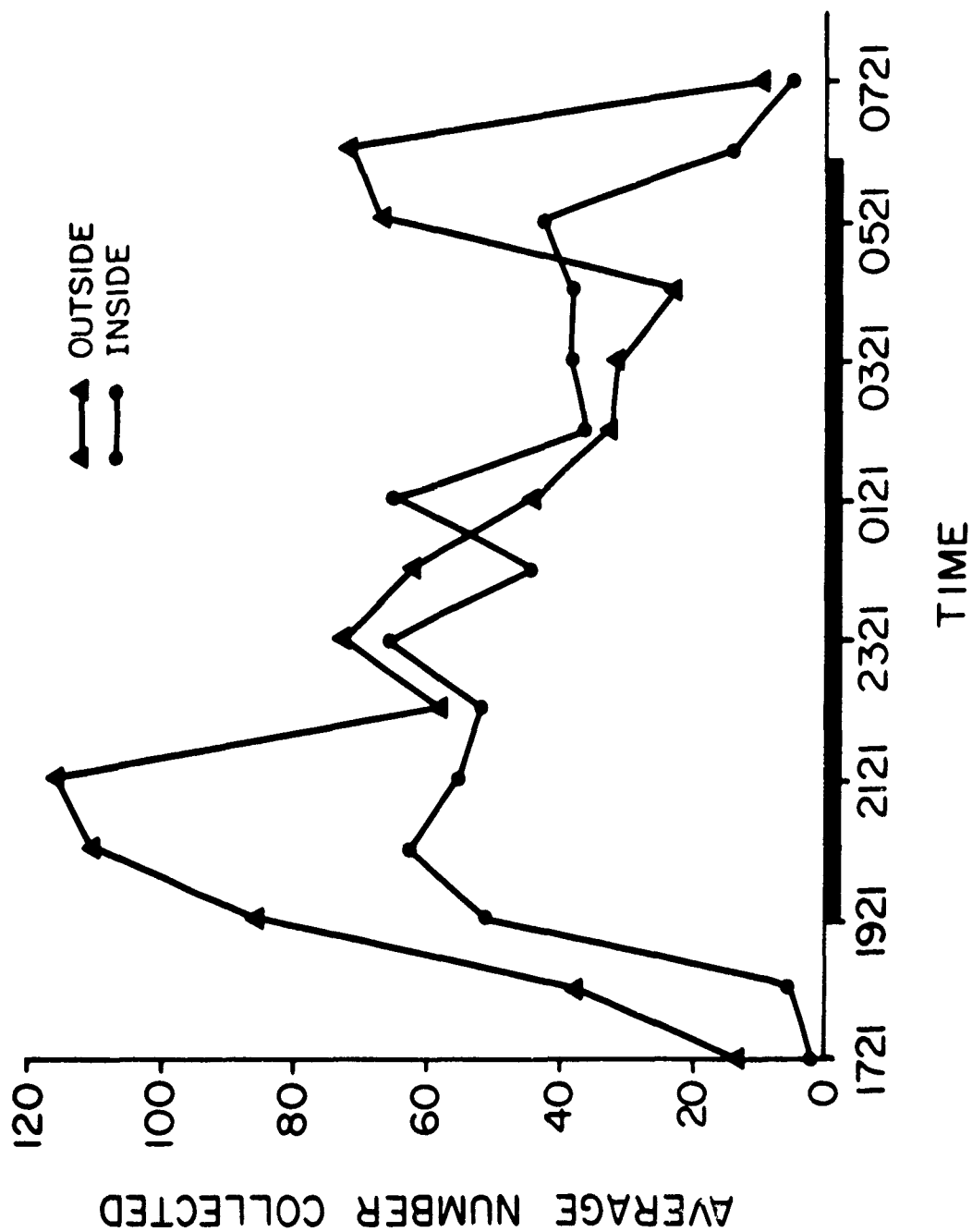


Figure 3. Numbers of Anopheles darlingi Root from 3 nights of human bait collections at Floresta, Ituxi River, Amazonas, Brazil in February 1979. Collections conducted by 1 collector each in the house and within 10 m of the house for 15 min. each hour (plotted by midpoint of collection intervals).

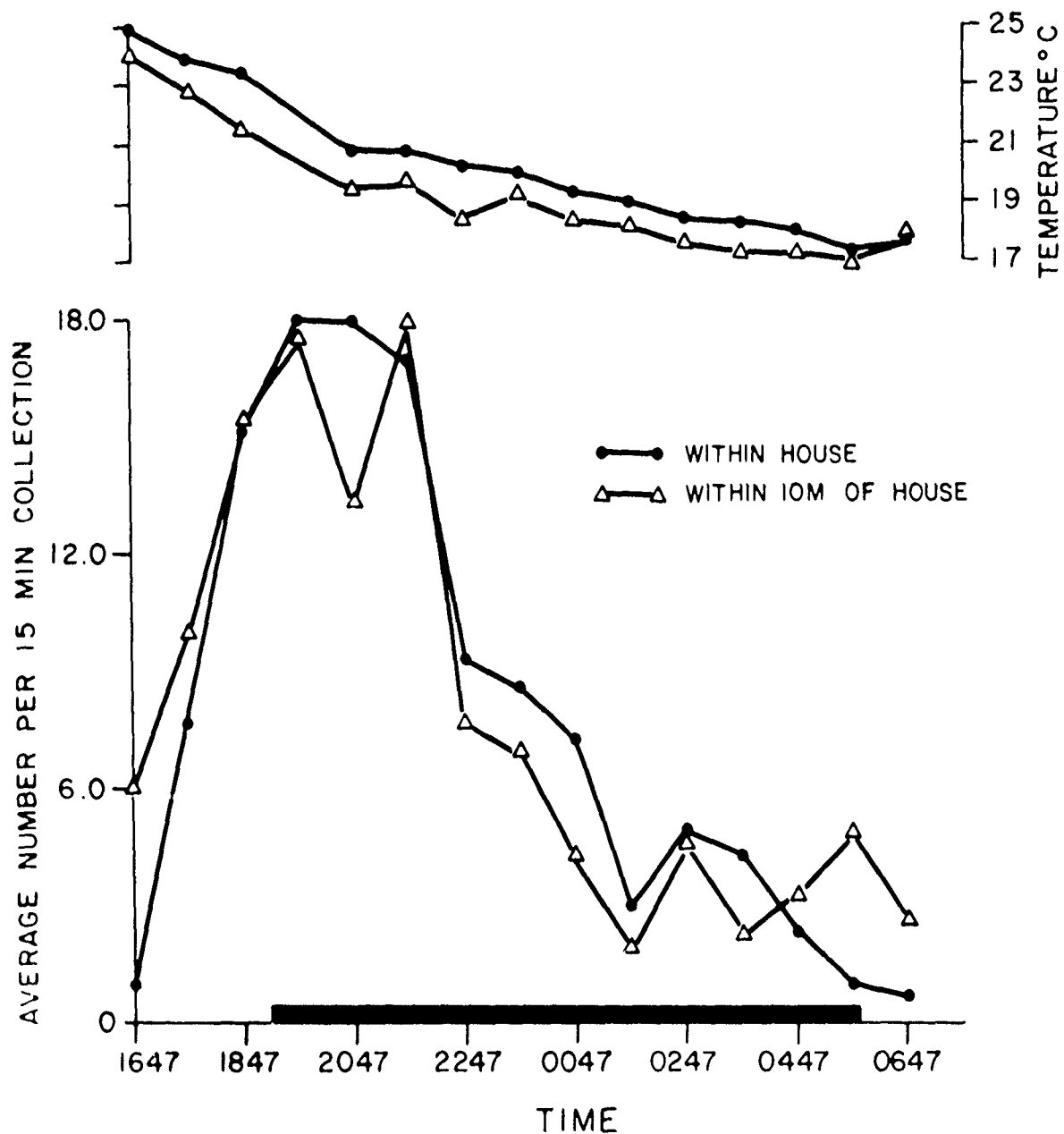


Figure 4. Numbers of *Anopheles darlingi* Root from 3 nights of human bait collections at Floresta, Ituxi River, Amazonas, Brazil, 31 May-3 June 79. Collections conducted by 1 collector each in the house and within 10 m of the house for 15 min. each hour (plotted by midpoint of collection intervals).

each collection. Objectives of this test procedure were to determine the relative contributions of collection time and temperature to numbers collected. The problem of endogenous activity rhythm influence on numbers collected by chronological time was minimized by testing 3 nights of sequential data from defined activity intervals. Our cumulative data revealed 4 activity intervals for An. darlingi in the peridomiciliary environment, viz., very low activity during the day, peak activity for about 3 hr during and after sunset, moderate to low activity during the 2200 - 0530 interval and a secondary peak of intense activity for < 30 min. at sunrise (0600 \pm 10 min). Therefore, separate tests were conducted on all collections from the 2 intervals, 1835 - 2055 and 2345 to 0500. Admittedly, the in-house activity patterns from study 1 did not reveal the 4 activity intervals as described. However, we believe data from outside biting collections reflect actual endogenous rhythms and that the process of seeking and gaining entry into the house from 1800 - 2200 hrs (Fig. 6) is another expression of peak activity at sunset. It seems likely that the continuous biting activity within the house results from the population responding to a different set of feeding "cues" than populations feeding outside the house.

The mechanics of the test procedure consisted of calculating separate Kendall Rank Correlation Coefficients for numbers collected *vs* temperature, numbers collected *vs* time and temperature *vs* time for data from both activity intervals. Tests of significance were performed on the r values at the 0.01 level of probability. The r values were then employed in the Kendall Partial Rank Correlation Coefficient to parcel out the time and time-temperature effects. No tests of significance are available for the resultant $r_{xy.z}$ values. Results of data analysis from both activity intervals are presented below. High r oe values for numbers collected with different temperatures indicate that temperature was the main determinant for number collected within the activity intervals.

Systematic collections outside the house were initiated during study 2, 3-7 June 1979, to characterize the level of biting activity during the day and to document the crepuscular peaks in biting activity with warmer ambient temperatures. The frequency of human bait collections were increased during the early morning and evening to more precisely document periods of peak activity. We verified the previously reported observation that the morning peak is intense and of short duration (Fig. 5). Also the early evening peak was duplicated in these series of collections. Data from collections conducted throughout the day demonstrated the absence of biting activity only in the early afternoon.

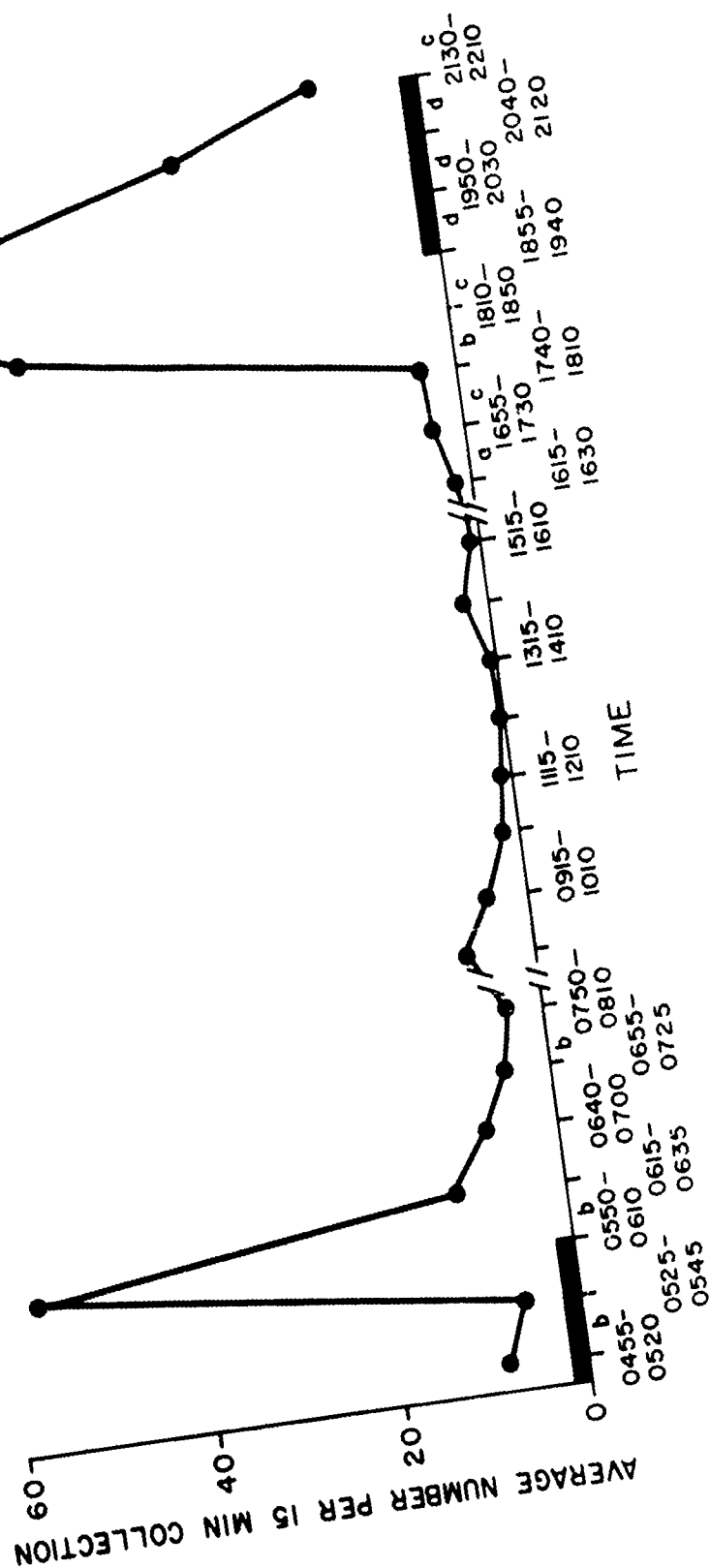
Results from entrance and exit trapping of darlingi during studies 1 and 2 demonstrated a surge of numbers entering the house from

Figure 5. Numbers of Anopheles darlingi Root from 2 days of human bait collections at Floresta, Ituxi River, Amazonas, Brazil in June 1979. All collections were conducted by 1 collector within 10 m of the house for 15 min each (plotted by time intervals).

- a Value based on a single 15 min collection.
- b Average from 3 collections.
- c Average from 4 collections.
- d Average from 5 collections.

TEMPERATURE °C

31
29
27
25
23
21
19



1514

Analysis of data from two activity intervals.

Activity Interval (time)	Variables	r	$r_{xy.z}$
1835-2055	r_{xy} = Number Collected vs. Temperature	0.70**	_____
	r_{xz} = Number Collected vs. Time	0.04	_____
	r_{zy} = Temperature vs. Time	0.01	_____

Kendall Partial Rank Correlation Coefficient = 0.70

2345-0500	r_{xy} = Number Collected vs. Temperature	0.64**	
	r_{xz} = Number Collected vs. Time	0.41	
	r_{zy} = Temperature vs. Time	0.53*	

Kendall Partial Rank Correlation Coefficient = 0.55

*

Significant at 0.01 level of probability ($p < 0.0027$).

**

Significant at 0.01 level of probability ($p < 0.0007$).

1800-2200 hours (Figs. 6 and 7). Exodus from the house did not begin until 0400 hours. Again, there were marked differences in the study 1 and study 2 collections results. The entrance of females peaked earlier (1800-2000) and was of short duration in study 2; in addition, movement out of the house started later (0600-0800) and continued through mid- to late-morning. These differences are perhaps another expression of the temperature influence on the activity of darlingi populations.

Based on data presented in Figures 6 and 7 it seems that darlingi enter the house in the evening, with peak activity between 1800 and 2200, remain in the house until sunrise and exit. Data from both studies indicate that very few specimens remain inside the house during the day and rarely did gravid females appear in the exit traps. The preponderance of late fed specimens in exit traps at

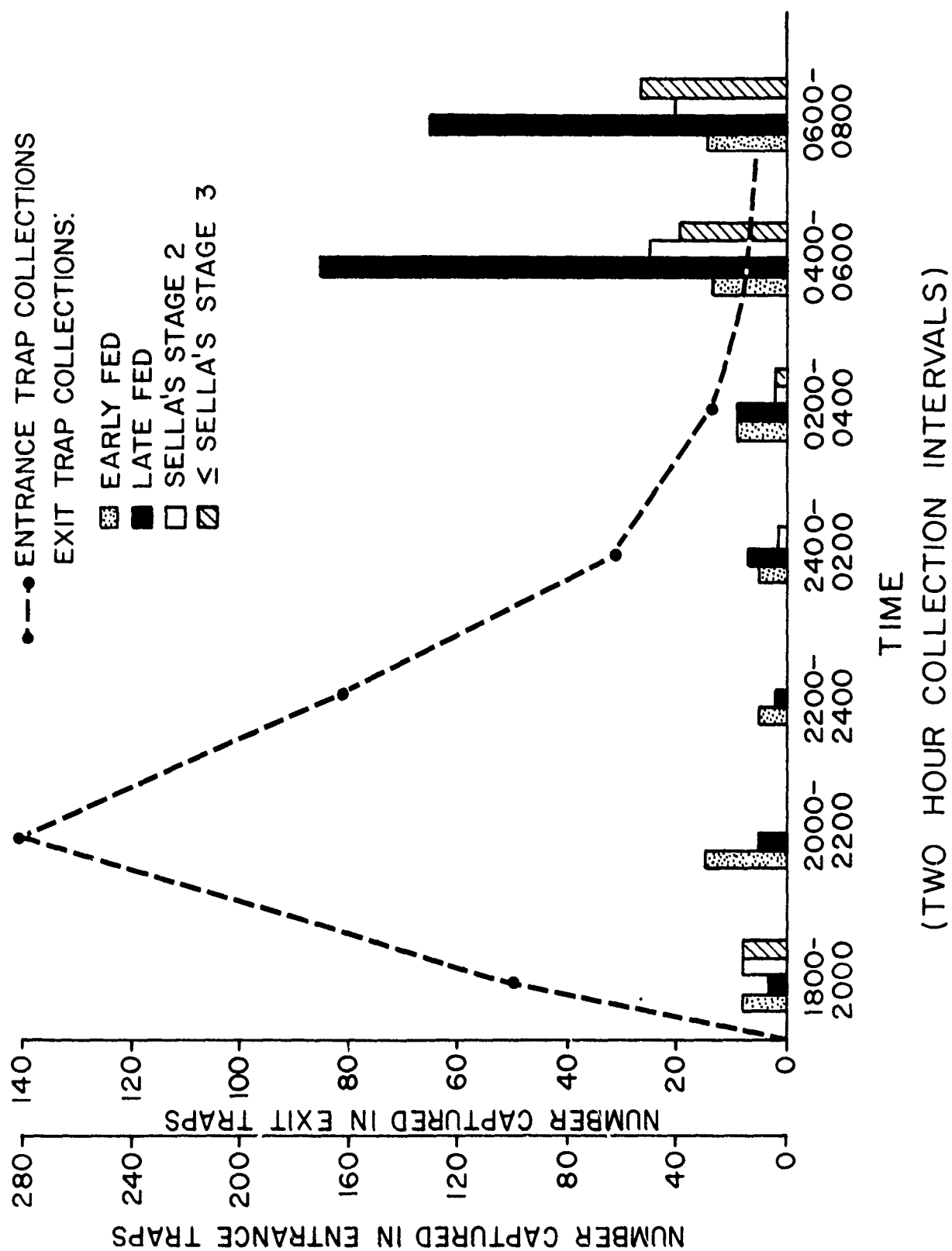


Figure 6. Numbers of *Anopheles darlingi* Root from trapping with 3 entrance and 3 exit traps at Floresta, Ituxi River, Amazonas, Brazil in February 1979.

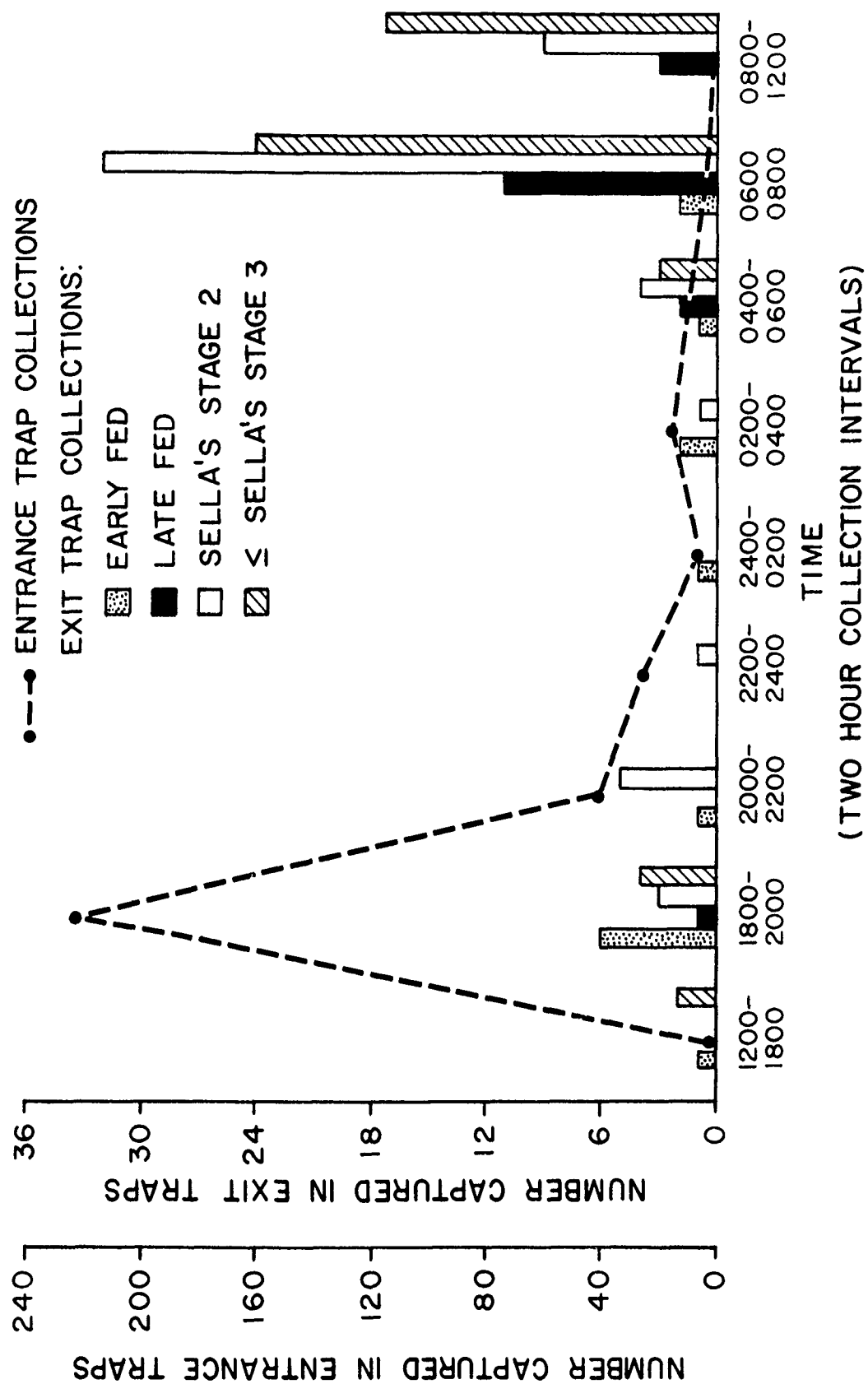


Figure 7. Numbers of *Anopheles darlingi* Root from trapping with 3 entrance and 3 exit traps at Floresta, Ituxi River, Amazonas, Brazil, 31 May - 3 June 1979.

sunrise in study 1 is compatible with a continuous pattern of biting activity throughout the night (Fig. 3). Whereas, the greater number of Sella 2 specimens, recorded in exit traps during study 2, results from the early evening peak in biting activity (Fig. 4).

Observations on the rate of blood digestion and ovarian development in 100 female An. darlingi were conducted during the study in February, 1979. The study specimens were maintained at ambient temperatures in the experimental house. All early fed females were clumped in the 1-5 hr. interval (Table 17). Specimens classified as late fed were found in both the 1-5 and 6-10 hr intervals; Sella 2 individuals were clumped in the 6-10 hr intervals and the majority (79%) of Sella 3 specimens were in the 11-20 hr interval. Since more Sella 3 specimens were in 6-10 hr interval than in 21-30 hr interval, it seems likely that most specimens attain this stage during the first part of the 11-20 hr interval.

Engorged An. darlingi, marked with fluorescent powder, were released inside the experimental house on 2 separate occasions during study 1 to determine their preferred resting sites. Periodic searches for marked specimens were made throughout the night. Although total numbers progressively declined with time after release, the majority of engorged darlingi were consistently found resting on the ceiling (Table 18). A search made outside the house prior to sunrise, revealed no particular preference for resting sites.

When unengorged specimens from exit trap collections were marked and released during study number 2, we again observed a preference for darlingi to rest on the ceiling (Table 19). This preference was particularly marked during the first 1.5 hours after release. There was a more equal distribution of numbers resting on the walls and ceiling later in the night. Again, we do not know if low temperatures recorded during study 2 influenced their selection of resting sites.

In addition to making observations on resting sites of marked, unengorged specimens released during study 2, we also tabulated numbers of marked specimens collected in exit traps (Table 20) and numbers collected in the hourly 15 min. human bait collections (Table 19 and Fig. 8). More than 50% of marked individuals collected from human bait were obtained during the first 3 hr after release (Fig. 8). In contrast, only 2 of 11

TABLE 17

Observations on the rate of blood digestion and ovarian development in *Anopheles darlingi* Root, at in-door temperatures and humidities*. Observations made with wild caught females on the Ituxi River, Amazonas, Brazil in February-March 1979.

Hours post-engorgement	Early fed	Late fed	Sella Stage						
			2	3	4	5	6	7	Undetermined
1 - 5	14	5							0
6 - 10		7	6	4					3
11 - 20				19					1
21 - 30				1	6				2
31 - 40					1	7	5		1
41 - 50						4		5	0
51 - 60								9	0
TOTALS	14	12	6	24	7	11	5	14	7

*

Temperature range limits were 24° - 3°C.

Relative humidity range limits were 80 - 100%.

TABLE 18

Numbers of marked* ♀♀ *Anopheles darlingi* Root observed by resting site at intervals throughout the night in an experimental house at Floresta on the Ituxi River, Amazonas, Brazil. Two tests were conducted (22-23 February and 28 Feb - 1 March 79) and 100 specimens were collected in human bait captures, permitted to engorge, marked and released for each study.

Hours after release	Numbers seen					
	Inside			Outside		
	Floor	Wall	Ceiling	Wall	Under floor	Under roof overhang
0.25 ^b	2	28	35	-	-	-
0.5 ^a	8	22	27	-	-	-
1.0 ^b	0	19	25	-	-	-
1.5 ^a	0	9	29	-	-	-
7.25 ^b	0	5	15	-	-	-
7.5 ^a	0	3	5	2	3	0
						2

* Females were marked with USR pigment 1953 and marked specimens were subsequently identified with a Blak-Ray, ULV;56, long wave ultra-violet lamp.

^a Observations were made on 100 specimens released 22 February, 1979.

^b Observations were made on 100 specimens released 28 February, 1979.

TABLE 19

Number of marked* ♀♀ *Anopheles darlingi* Root observed by resting site at intervals throughout the night in an experimental house located at Floresta on the Ituxi River, Amazonas, Brazil. A total of 81 and 82 marked, unfed specimens were released 1 and 2 June 1979, respectively.

Hours after release	Number resting by site within the house				Totals
	Floor	Walls	Rafters	Ceiling	
0.5 ^a	5	14	7	28	54
1.0 ^b	0	10	4	27	41
1.5 ^a	0	10	3	25	38
\bar{X}	1.7	11.3	4.7	26.7	44.3
4.5 ^a	0	14	0	13	27
5.0 ^b	0	10	1	7	18
\bar{X}	0	12	0.5	10	22.5
8.5 ^a	0	7	0	11	18
8.5 ^b	0	7	0	6	13
\bar{X}	0	7	0	8.5	15.5

* Females were marked with USR pigment 1953 and marked specimens were identified with a Blak-Ray, ULV;56, long wave ultra-violet lamp.

a Observations made on the 81 specimens released 1 June 79.

b Observations made on the 82 specimens released 2 June 79.

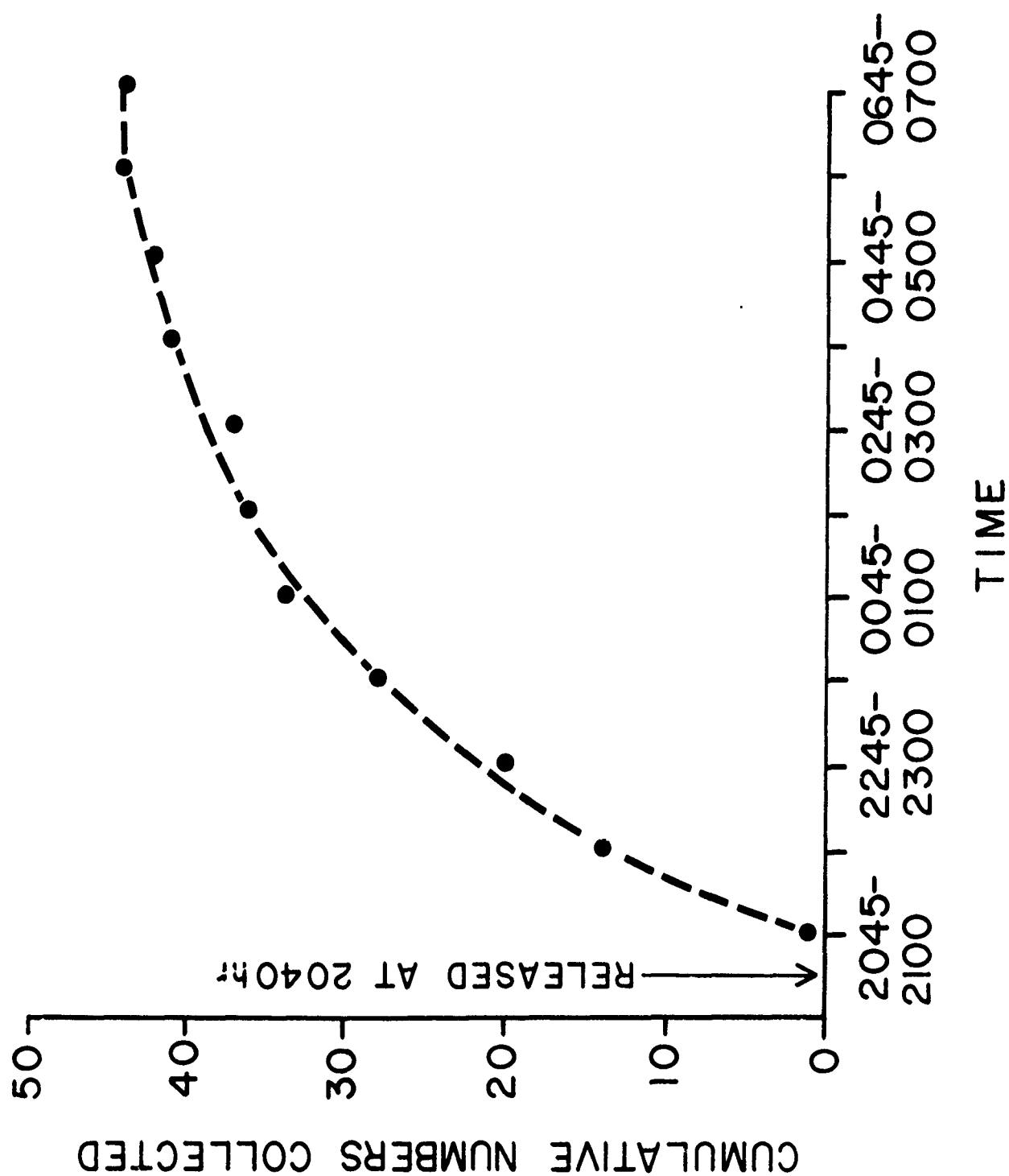


Figure 8. Cumulative numbers of marked *Anopheles darlingi* (out collected in human bait collections followin release inside a house at Floresta, Ituxi River, Brazil, at 2040 hr. A total of 81 and 82 marked, unfed specimens were released 1 and 2 June 1979, respectively.

marked specimens that were captured in the exit traps were collected before 0600 hours (Table 20). After chronological adjustments were made in numbers of marked specimens, following removal by trapping (Table 21), we found a cumulative 32.9% of marked specimens were captured in human bait collections and 9.5% in exit trap collections.

The mark-recapture data, obtained during this study, is interpreted cautiously for the following reasons: a) very small numbers were recorded from the exit trap collections; b) marked specimens were released late in the period of peak biting activity (2045 hr); c) abnormally low ambient temperatures were recorded during both study nights; and d) the marked populations were discrete and do not reflect the variable but continual immigration and emigration of darlingi within the house.

The impact of these factors can be seen in the values of marginal totals in Table 20. The last column reveals that immediately following release, the marked specimens are more abundant in the exit trap collections than unmarked individuals, whereas engorged females are more abundant in subsequent collections. In addition, there were disproportionately few marked early to late fed specimens, a disproportionately large representation of unfed specimens (Sella 1), a proportionate number of Sella 2 and 3 specimens and no marked specimens in stages > Sella 3 (see marginal totals in last row of Table 20). This reflects the discrete characteristic of the marked population in that some exit immediately, none are in the house a sufficient time to be > Sella 3 and most feeding took place immediately, thus marked individuals were in Sella 2 and 3 at sunrise.

Calculations with the simple Lincoln Index (24) were performed to estimate total numbers of An. darlingi in the house with human bait and exit trap collection data. For these calculations, a = total number marked and released, n = number collected (marked + unmarked) after release, y = number recaptured after release and p = total number of darlingi in the house. The p value is calculated with the Lincoln Index formula:

$$p = \frac{an}{r} .$$

The a value was determined by multiplying the accumulative % collected by the number of marked specimens released (Table 2), e.g., for human bait collections it is $0.329 \times 163 = 53.6$ and for exit trap collections it is $0.095 \times 163 = 15.5$.

Calculations with the human bait collections gave a total of 504.8 An. darlingi in the house for the 2 nights ($n = 166$, $a = 163$ and $r = 53.6$). The estimate of populations size was 557 darlingi with

TABLE 20

Number of marked * ♀♀ Anopheles darlingi Root recaptured in 3 exit traps following release inside and experimental house on the Ituxi River at Floresta, Amazonas, Brazil. Combined data from releases of 81 and 82 marked, unfed specimens at 2040 hr on 1 and 2 June 1979, respectively.

Time	Early		Late		Sella stage							Total number captured (marked/unmarked)
	fed		fed		1	2	3	4	5	6	7	
2000-2200	0/0		0/0		2/0	0/1	0/0	0/0	0/0	0/0	0/0	2/1
2200-2400	0/0		0/0		0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/1
0000-2000	0/1		0/0		1/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1
0200-0400	0/0		0/0		0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
0400-0600	0/0		0/0		0/0	0/2	0/1	0/0	0/0	0/0	0/0	0/3
0600-0800	0/0		1/6		2/4	1/3	2/6	0/2	0/0	0/0	0/1	6/22
0800-1200	0/0		0/1		0/0	2/2	1/2	0/0	0/0	0/0	0/0	3/5
Total number captured (marked/unmarked)	0/1		1/7		4/4	3/9	3/9	0/2	0/0	0/0	0/1	11/33

* Females were marked with USR pigment 1953 and marked specimens were identified with Black-Ray, ULV; 56, long wave ultra-violet lamp.

TABLE 21

Number available, number collected, % collected and accumulative % collected of marked * ♀♀ Anopheles darlingi Root in human bait and exit trap collections in an experimental house at Floresta, Ituxi River, Amazonas, Brazil. Combined data from releases of 81 and 82 marked, unfed specimens at 2040 hr on 1 and 2 June 1979, respectively.

Time	Human bait collections			Exit trap collections			
	Number available	Number collected	% collected	Accumulative %	Number available	Number collected	Accumulative %
2000-2200	163	16	9.8	9.8	147	2	0.14
2200-2400	145	15	10.3	20.1	130	0	0
2400-0200	130	8	6.2	26.3	122	0	0
0200-0400	122	5	4.1	30.4	117	0	0
0400-0600	117	3	2.6	32.9	114	0	0
0600-0800	113	0	0	32.9	113	6	5.3
0800-1200	106	-	-	-	107	3	2.8
1200-1800	103	-	-	-	103	0	0
							1.4
							1.4
							1.4
							1.4
							1.4
							6.7
							9.5
							9.5

*

Females were marked with USR pigment 1953 and marked specimens were identified with Black-Ray, ULV;56, long wave ultra-violet lamp.

Number of *Anopheles darlingi* Root in human bait collections conducted at 3 sites near an experimental house at Floresta, Ituxi River, Amazonas, Brazil. Collections were conducted for 15 min. each at all 3 sites simultaneously, 3-4 June 1979.

* Collection site located in a low secondary forest.

TABLE 23

Species and numbers of specimens captured in human bait collections conducted simultaneously at 3 sites (10 collections/site) near an experimental house at Floresta, Ituxi River, Amazonas, Brazil. Collections were conducted for 15 min. each from 1740-2005, 3 and 4 June 1979.

Distance from experimental house		
10 meters	20 meters	40 meters
<u>Anopheles (Nyssorhynchus)</u> <u>darlingi</u> (539)	<u>Anopheles (Nyssorhynchus)</u> <u>darlingi</u> (106) <u>nuneztovari</u> (21) <u>oswaldoi</u> (19) (<u>Anopheles</u>) <u>peryassu</u> (2) <u>mediopunctatus</u> (19) <u>shannoni</u> (1) <u>Aedes</u> <u>fulvus</u> (2) <u>Psorophora</u> <u>cingulata</u>	<u>Anopheles (Nyssorhynchus)</u> <u>darlingi</u> (78) <u>nuneztovari</u> (4) <u>oswaldoi</u> (3) (<u>Anopheles</u>) <u>peryassu</u> (1) <u>mediopunctatus</u> (7) (<u>Stethomyia</u>) <u>nimbus</u> (1) <u>Aedes</u> <u>fulvus</u> (2) <u>Culex</u> <u>spisspes</u> (1) <u>Psorophora</u> <u>cingulata</u> (3)

TABLE 24

Number of ♀♀ *Anopheles darlingi* Root collected in 5 min. resting captures inside and outside of an experimental house at Floresta, Ituxi River, Amazonas, Brazil. Collections were conducted by 1 collector each 28 February - 1 March 1975.

Time	Number found resting by site		
	Inside house wall	Outside house wall	Vegetation
1830-1835 ^a	0	0	0/1
1830-1835 ^b	0	1/0	0/1
1900-1905 ^a	0	0	0/1
1900-1905 ^b	0/3	1/0	0/1
1930-1935 ^a	0	0/3	0/2
1930-1935 ^b	0/1	0/7	0/5
2000-2005 ^a	1/0	0	0/12
2000-2005 ^b	1/0	0/2	0/2
2030-2035 ^a	2/0	0/4	0/1
2030-2035 ^b	1/0	0/3	2/6
2100-2105 ^a	1/0	0	0/2
	6/4	2/19	2/34
0540-0545 ^b	1/0	0/1	2/0
0610-0615 ^b	0	0	1/0
0640-0645 ^b	0	0	1
0710-0715 ^b	1/0	0	0
	2/0	0/1	3/0

^a Collections conducted 28 February 1979.

^b Collections conducted 1 March 1979.

exit trap data ($n = 53$, $a = 163$ and $x = 15.5$). The similarity between these population estimators is interesting but does not prove degree of accuracy. Additional studies are required to fully understand the variables involved with this study method.

Collections to study the temporal and spatial distribution of An. darlingi away from the house during the early evening activity interval were conducted during study number 2. Collections were conducted for 15 min, each at different distances from the house, viz., < 10, 20 and 40 meters from the house. Prior to sunset the greatest number were collected furthest from the house, but all subsequent collections were uniformly high near the house compared to the more remote collecting sites (Table 22). The crepuscular peak in activity was clearly revealed near the house, but not at 20 m and 40 m from the house. Only An. darlingi were collected near the house, whereas we observed a considerable increase in species diversity and a great decrease in numbers of darlingi at 20 m and 40 m from the house (Table 23).

Collections of resting darlingi were conducted inside and outside the house from 1830 - 2105 and 0540 - 0715. The females collected inside the house prior to 1935 hr were unfed, but all subsequent specimens were engorged (Table 24). The majority of specimens caught outside were unfed during the evening. However, three of 4 specimens collected resting outside in the morning were engorged.

c. The Role of Euglossine Bees in the Removal of DDT from Sprayed Houses.

1) Objectives: To determine the impact of prolonged bee activity on the residue levels of DDT on walls of sprayed houses.

2) Background:

The strong insecticidal activity of DDT (dichlorodiphenyltrichloroethane) was first demonstrated in 1943 (24). DDT was subsequently employed throughout the world as a "front line defense" against insects of agricultural or public health importance, and is still widely used for residual treatment of house walls for control of mosquitoes in Brazil.

While studying the ecology of the malaria vector Anopheles (Nyssorhynchus) darlingi Root along the Ituxi River, Amazonas, Brazil, we observed aggregates of male bees on the walls of houses that are routinely treated with DDT. The possibility of DDT being an insect attractant is incongruous with its premier role as an insecticide. Thus, our curiosity was aroused when we discovered that these Euglossine bees, identified as Euplusia purpurata, were

well known to the residents as the insects that eat DDT ("o bicho que come DDT").

The euglossine bees have been the subject of many fascinating studies due to their important role as plant pollinators. Both males and females provide valuable pollination services in their pursuit of nectar. An even more specific relationship has been found for the males in their apparent pursuit of certain flower odors, i. e., many flowers attract only one species of bee that affects pollination (26). The flower visiting behavior of the males has been described as follows:

"When they visit an euglossine flower, they brush on the surface of the flower with pads of hair on the forefeet. The bees characteristically brush for a short time and they hover near the flower while scrubbing their legs together and evidently placing some substance in their inflated hind tibiae. The bees usually repeat this behavior several or many times, sometimes remaining as long as 90 minutes at one inflorescence" (26).

3) Progress:

We collected several bees while they were busily brushing their foretarsi on the DDT-treated walls of houses. Thirty or more constantly active bees would be seen at one time, and as some would leave, others would enter. The occasional presence of very shallow grooves at the site of bee activity indicated that perhaps the heavily sclerotized mandibles were sometimes employed to collect the DDT residues. The residents stated that these bees appear after houses are sprayed with DDT and are most abundant immediately after treatment.

Five of the bees we collected were sent to the U.S. Army Environmental Hygiene Agency, Aberdeen Proving Ground, Md., where they were dissected and each body part separately analyzed for DDT residues in accordance with analytical procedures recommended by the USEPA (27). The head, thorax, abdomen and fore-, mid- and hindlegs were processed for each specimen (wings were discarded). The body parts were weighed, prior to DDT extraction, so concentrations of insecticide could be expressed in parts per million (ppm) by body weight.

Total residue in $\mu\text{g}/\text{bee}$ for 5 DDT isomers are presented in Table 25. Clearly the p,p' - DDT isomer was present consistently in the greatest concentrations. The sum of the DDT isomers expressed

TABLE 25

DDT residues from 5 males of Euplusia purpurata collected from sprayed houses along the Ituxi, River, Amazonas, Brasil.

DDT	Residue concentrations (ub/bee) for 5 DDT isomers				
	Bee No.1	Bee No.2	Bee No.3	Bee No.4	Bee No.5
o,p' - DDD	2.32	4.59	0.80	*	*
p,p' - DDD	24.99	69.47	44.01	32.49	80.45
p,p' - DDE	16.02	52.24	36.38	28.64	58.84
p,p' - DDT	135.25	472.53	174.70	11.21	335.95
p,p' - DDT	985.80	2944.72	2087.35	1545.50	2630.54
DDTR	1169.32	3557.95	2352.50	>1654.81	>3122.06

*

Part of the extract lost during centrifugation in the laboratory.

as DDTR*, converted to parts per million by dry weight, revealed exceptionally high concentration of DDT in these Brazilian bees (Table 26). Highest concentrations were consistently found in the hindlegs, with smaller amounts in the fore- and midlegs. Although significant residue levels were found in head, thorax and abdomen, DDT concentrations in these regions were markedly less than what was found in the hind-legs. For honey bees, the average LD₅₀ (lethal dose for 50% of test populations) for p,p' - DDT alone is 5.6 ug/bee (28). Although it is inaccurate to compare values from one species to another, these statistics provide a perspective for evaluating the high residue levels found in the Brazilian bees. Looking at total p,p' - DDT in ug/bee, for instance, there was a low of 985 in bee 1 and a high of 2944.72 in bee 2, or 176.0 to 525.8 times greater than the LD₅₀ value for honey bees. Since no dead or moribund bees were seen at the collections sites, the high residue levels of DDT suggest a high degree of resistance in these euglossine bees. Bees included in this analysis were collected at 3 isolated houses separated by 1-2 days travel by boat and it seems certain that the DDTR residue found in the bees was a direct result of contact with the sprayed house walls.

We have considered the possibility that males of E. purpurata enter houses because of attractants in the house construction materials or in response to carriers in the DDT formulation. For the latter, it is relevant to note that attractants to male euglossine bees are characteristically aromatics (28, 29). The DDT sprayed in houses along the Ituxi River is applied as a wettable powder formulation, composed of DDT, talc and water. Clearly, DDT is only aromatic in this mixture. In addition, males of E. purpurata have been observed scratching on boards treated with another aromatic insecticide, Aldrin (29). Regarding the possibility of attractants in house construction materials, houses along the river are constructed mainly of local forest products, viz., deciduous trees for the frame, palm thatching for the roof and palm slats for the walls and floors. Although the bees seen to prefer the ceiling, they frequently observed brushing on all types of house construction materials. In one case, they were found brushing on a tree trunk, in the forest, that had been spot-sprayed with DDT.

Bees have been reported to enter houses after DDT applications by residents near Manaus, Amazonas, Brazil. They also have been

*

DDTR is a means of summing all the DDT isomers and expressing the total in terms of DDT. The following equation is utilized:

$$[(o,p' + p,p' - DDD) + (o,p' - p,p' - DDE)] + 1.114 + (o,p' + p,p' - DDT).$$

TABLE 26

Residue levels of DDTR in male Euplusia purpurata collected from sprayed houses along the Ituxi, River, Amazonas, Brazil.

Bee No.	Residue levels of DDTR in ppm or ug/g						Total ppm ug/g/bee
	Forelegs	Midlegs	Hindlegs	Head	Thorax	Abdomen	
Bee No. 1	451.87	930.14	10,526.15	123.58	29.95	118.78	12,180.48
Bee No. 2	1,266.54	4,245.42	29,082.55	369.02	60.47	203.15	35,227.14
Bee No. 3	2,464.81	6,222.27	11,203.44	839.04	153.87	121.08	21,004.
Bee No. 4	2,768.94	1,685.38	10,631.66	—	99.11	—	15,185
Bee No. 5	10,997.78	3,706.23	25,025.52	1,326.07	399.26	172.65	41,627.52

observed brushing on house walls near Iquitos, Columbia (personal communication, Dr. R. L. Dressler, Smithsonian Tropical Research Institute, Box 2072, Balboa, Canal Zone). That this is a wide-spread phenomenon was further verified by a recent report on the sightings of E. purpurata within treated houses in many areas in the state of Para, Brazil (30). However, no documentation of these insects actually collecting insecticide has been made and this is the first such report.

Based on the distribution of DDT residues in the bees, it seems that they do not seek the insecticide for strictly dietary purposes. Thus, we suspect that E. purpurata males are attracted by the odor of DDT which then stimulates their behavior of brushing the insecticide from walls for storage in a pouch of the hind tibia (saccate hind tibial organ).

d. Comments:

In summary, we have documented exophilic and endophagic behavior patterns for An. darlingi populations at the Ituxi River study area. Even in the unsprayed experimental house at Floresta the An. darlingi do not rest inside the house for more than a few hours. Additional observations are as follows:

- 1) Anopheles darlingi are consistently present at most of the single and multiple family habitations along the Ituxi River systems;
- 2) The An. darlingi populations demonstrate peak host-seeking activity at sunset and sunrise in the peridomiciliary environment;
- 3) Activity, in the absence of perturbations from low ambient temperatures, within a house with complete walls is relatively constant throughout the night;
- 4) Biting activity in a partially enclosed house (with one wall) reflects the bimodal pattern documented for the peridomiciliary environment;
- 5) The spatial distribution of host-seeking darlingi populations is clumped near to and within the experimental house;
- 6) The preferred, within house, resting site of engorged An. darlingi is on the ceiling.

The latter observations is in sharp contrast to the finding of Deane and Damasceno that 87.2% of the An. darlingi collected were resting on the lower 2 m of the house walls. Since walls are frequently not over 2 m in height, with consequent large openings between the roof

(

and walls, selection of the ceiling resting sites may facilitate escape from the house. Also it is interesting that E. purpurata seem to preferentially collect DDT (see section c) from the ceiling and upper levels of the wall.

The objective for future studies is to determine the influence of DDT treatment on the behavioral parameters described in this report. In some cases we will seek further verification of our observations prior to spraying the experimental house with DDT. However, a control house, that will not be sprayed, is under construction. It is important to identify the main points of entry and exit of An. darlingi from the house and to elucidate the variability in activity as a result of different portals of exit. More emphasis will be placed on studies of resting sites and host preferences. These efforts will be facilitated by the employ of a recently constructed vacuum aspirator. More detailed studies will be conducted to determine a) the impact of the euglossine bees in DDT removal and b) characterize, in more detail, their insecticidophilic behavior.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL
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11. TITLE (Provide with Security Classification Code) ^a							
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12 SCIENTIFIC AND TECHNOLOGICAL AREA ^a							
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19 RESPONSIBLE DOD ORGANIZATION				20 PERFORMING ORGANIZATION			
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Pursuant to FAR 101.11 U.S. Academic Institution)			
NAME: Russell, Philip K. COL				NAME ^a LeDuc, J.W. MAJ MSC			
TELEPHONE: 202-576-3551				TELEPHONE: 226-1557			
				SOCIAL SECURITY ACCOUNT NUMBER			
21 GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence not considered.				NAME: Hoch, A.L. CPT MSC			
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22 KEYWORDS (Provide EACH with Security Classification Code) (U) Virology; (U) Entomology; (U) Epidemiology; (U) Mammalogy; (U) Ornithology; (U) Ecology; (U) Brazil.							
23. (U) Conduct studies on the ecology of Oropouche (ORO) virus transmission in the Amazon basin in collaboration with the Evandro Chagas Institute. This arbovirus is the most common cause of arbovirus epidemics in the region and commonly results in febrile, debilitating illness. Information on the dynamics of ORO virus transmission in urban and sylvatic environments is of importance to military personnel transiting or stationed in this geographic area.							
24. (U) Special and routine virological, entomological and epidemiological procedures were employed. Field collected specimens of insects, bird and mammal blood and organs will be processed for virus isolation. Sera will be tested for antibodies to ORO virus to detect areas of recent ORO virus activity.							
25. (U) 78 10- 79 09 Investigations were terminated by mutual consent of the Brazilian Ministry of Health, the Institute Evandro Chagas and the Walter Reed Army Institute of Research. Termination of this project was prompted by the failure of the Brazilian Ministry of Health to renew a standing agreement which authorized scientific investigations in Brazil. For final technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 October 1978-30 September 1979.							

PROJECT 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit No. 010 Field Studies of Sandfly Fever Viruses

Investigators:

Principal: Major James W. LeDuc, MSC and Jack W. Millar, M.D.

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INTRODUCTION AND SUMMARY OF ACCOMPLISHMENTS

In the spring of 1979 a cooperative program was established between the Walter Reed Army Institute of Research (WRAIR) and the Gorgas Memorial Institute (GMI). This program allowed a WRAIR scientist to conduct studies in Panama while utilizing the Laboratory facilities at the Gorgas Memorial Laboratory, Panama. The WRAIR scientist was given the rank of Adjunct Associate within the GMI structure and functions essentially as a GMI staff member. Funding for research is provided through WRAIR.

The initial topic of study for the WRAIR-GMI program is to determine the incidence and prevalence of Phlebotomus fever group viruses in Panama, with special emphasis being placed on the impact of these agents on the military population stationed there. The study design for this project calls for close monitoring of 4 military units permanently assigned to Panama which frequently engage in field exercises. These units have a combined total strength of approximately 2500 individuals. Blood samples will be collected from all individuals assigned to the units under study as they rotate in and out. These sera will be examined for the presence of antibody to Phlebotomus fever group viruses in an effort to determine the prevalence of antibody in this high exposure population. An attempt will also be made to monitor episodes of acute illness among individuals assigned to these units, and to isolate virus from febrile patients.

Preliminary serological results are available for 2 of the 4 units under study. Of 484 serum samples collected from people assigned to the 4th BTN, 10th INF, Fort Davis, one (0.2%) had antibody to Chagres virus, a Phlebotomus fever group virus known to cause human illness. An additional 22 (4.5%) had equivocal results, while the remainder lacked antibody. Of 158 sera from this same population tested for antibody to Punta Toro virus, another known human pathogen of the group, 3 (1.9%) had antibody, while the remainder were negative.

duration. His principle signs and symptoms included headache, dizziness, chills and fever. The patient has made an uneventful recovery, with no apparent sequela.

It is apparent from these preliminary results that both Chagres and Punta Toro viruses are infecting US military forces in Panama. In addition, this study has already documented that Chagres virus is responsible for at least some of the overt illness seen in this population.

Of 495 sera collected and tested from individuals assigned to the 4th BTN, 20th INF and associated units at Fort Clayton, 2 (0.4%) had antibody to Chagres virus, and an additional 28 (5.6%) gave equivocal results. The remainder were negative. No samples have been tested for antibody to Punta Toro virus from this population.

Only 4 acute samples have been collected and tested from clinically ill persons assigned to the units under study. From these, one sample has yielded Chagres virus. This patient experienced a febrile illness of approximately 4 days

EPIDEMIOLOGICAL STUDIES OF PHLEBOTOMUS FEVER VIRUSES

A. Review of the Literature

OBJECTIVE: The objective of this section is to present a succinct summary of the literature which deals with *Phlebotomus* fever group viruses. This summary will serve as a preface to the studies reported in the following sections.

BACKGROUND: The history of *Phlebotomus* fever (PF) has been documented primarily by the military since armies, rather than resident populations, have been the principle sufferers of the disease. In 1909 an Austrian Military Commission of Doerr et al. first demonstrated that the causative agent of sandfly fever was a virus transmitted by the sandfly, *Phlebotomus papatasi*. Subsequently Brit (1910) discussed *Phlebotomus* fever in Malta and Crete, while British experiences in India were later documented by Sinton (1925) and Anderson (1939). A very detail description of the American Military experience with sandfly fever was provided by Hertig and Sabin (1964).

Tesh et al. (1975) have provided the most recent and complete analysis of the PF group arboviruses in terms of antigenic relationships. In their presentation they provide complement fixation (CF), plaque reduction neutralization (PRN) and hemagglutination inhibition (HI) test results from experiments in which 21 known or presumed PF group viruses and 2 ungrouped viruses were considered. Included in these 21 virus are 6 serotypes described for the first time.

Since the publication by the Tesh et al. (1975), additional PF group viruses have been described. At present, 23 viruses have been formally registered and are considered to be distinct viruses within the group, and an additional 2 (Sud An 754-61 and Co Ar 3319) are still under consideration.

Members of the PF group of arboviruses are found throughout most of the world. The greatest diversity of serotypes within the group seems to be concentrated in the New World tropics, where 8 serotypes have been described from Panama and 7 from Brazil. In contrast, only 1 serotype has been described from the United States (Rio Grande virus). In the Old World, 4 serotypes are prevalent in the Mediterranean Region, and 4 are found predominantly in Africa. A clinical syndrome referred to as "*Phlebotomus* fever" has been reported from China (Bolt, 1915), but little mention has been made of it in recent western literatures.

Phlebotomus fever group viruses were not found to be impor-

tant human pathogens during the Viet Nam conflict, and no strains have been described from south east Asia. Likewise, PF group viruses have not been identified in Australia (Berge 1975; Knudsen et al. 1979).

A summary of the known geographic distribution of PF group viruses is provided in Table 1 .

Among the 24 known or presumed members of the PF group viruses, 5 are currently known to cause overt illness in man. These viruses are sandfly fever, Naples (SFN), sandfly fever, Sicilian (SFS), Chagres (CHG), Punta Toro (PT) and Candiru (CDU) viruses. All available information indicates that the clinical syndrome which results from infection with any of these 5 viruses is similar. Details of that syndrome will be presented in the following section.

Serological surveys have detected antibody to 13 PF group viruses. Besides the 5 viruses already mentioned that have been associated with overt illness, antibody to 8 others has been identified among naturally infected human populations. Investigations have failed to detect antibody to an additional 6 PF group viruses, and no data is available for the remaining 5 viruses. A summary of these observations is presented in Table 2 .

The clinical illness produced by infection with PF group viruses is thought to be essentially the same, regardless of which virus has caused the infection. The disease is characterized by sudden onset, fever lasting about 3 days ranging from 38-40°C (100-105°F), with severe frontal headache, pain in the eyes, back and joints, photophobia and general malaise (Sabin 1951; Hertig and Sabin 1964; Srinongse and Johnson 1974; Bartelloni and Tesh 1976). The disease is self limiting with complete recovery; no deaths have been attributed to infection with PF group viruses.

There is evidence to suggest that the clinical manifestations of PF group viruses may vary with age. In susceptible adults, experimental infection with Naples or Sicilian virus produces classical sandfly fever as described above (Sabin 1951; Bartelloni and Tesh 1976). Several studies have suggested that the disease in children is somewhat milder (Peschle 1936; Taylor 1959; Guelmino and Jertic 1955). Tesh et al. (1976) discuss this phenomenon in their report on sandfly fevers in the Old World. Results of serological surveys have shown that in areas where sandfly fever is endemic, most of the population is probably infected during childhood (Tesh et al. 1976; Saidi et al. 1977). Persons infected as children probably suffer only a relatively mild illness. In contrast,

when non-immune adults enter an endemic area, classical sandfly fever usually results.

Experimental infections of human volunteers with Naples and Sicilian sandfly fever viruses have demonstrated that a single infection with either virus confers immunity to the homologous virus type (Hertig and Sabin 1964; Bartelloni and Tesh 1976). Individuals subsequently challenged with the heterologous virus did, however, become infected.

Serological tests using convalescent sera from patients experimentally infected with either Naples or Sicilian sandfly fever virus had little or no neutralizing antibody to heterologous Old World PF group viruses (Bartelloni and Tesh 1976).

Neutralization tests of human sera collected from residents of Athens, Greece in 1973-75 and examined for antibody to PF group viruses found antibody to Sicilian virus only among persons over the age of 30 years, and to Naples virus only among persons over the age of 10 years. This distribution of antibody to PF group viruses by the age corresponds to the onset of insecticide usage for malaria control, and most likely reflects the effect of such spraying on the abundance of insect vectors of PF viruses. It also provides evidence which indicates that PF neutralizing antibodies persist for at least 30 years, and suggests that a single infection probably confers life-long immunity against the homologous virus (Tesh and Papaevangelou 1977).

Tesh and coworkers (1975) have presented the most complete analysis of the laboratory characteristics of the PF group viruses. They have examined nearly all recognized PF group viruses by HI, CF and PRN tests. Their results indicate that the PRN test is by far the most sensitive and specific test for both identification of isolates and detection of antibody. The PRN test has subsequently been successfully employed in a number of serosurveys to determine antibody prevalence rates to PF group viruses from various parts of the world (Tesh et al. 1976; Saidi et al. 1977).

The HI test has been used on occasion in the past for serosurveys, but results of these surveys must be interpreted with caution. Experimental infection of humans with Sicilian sandfly fever virus has shown that while a fourfold or greater rise in HI antibody titer followed infection, pre-infection sera frequently produced nonspecific inhibition of Sicilian antigen at dilutions from 1:10 to 1:40, despite prior treatment with Kaolin (Bartelloni and Tesh 1976).

The CF antibody response of volunteers infected with Sicilian

sandfly fever virus was generally poor. CF antibody titers in convalescent sera varied from 1:4 to 1:16, with most in the 1:4 to 1:8 range (Bartelloni and Tesh 1976).

Eight PF group viruses have been identified from Panama. These are: Chagres; Punta Toro; Cacao; Chilibre; Aguacate; Nigue; Caimito; and Frijoles viruses. Of these, Chagres and Punta Toro viruses have both been isolated from clinically ill persons, and are thus recognized as human pathogens (Peralta et al. 1965; Berge 1970). Antibody to Cacao virus has been identified among people resident in Panama, but no documentation has been made of clinical illness associated with infection by this virus (Berge 1975). Antibody was not found to either Aguacate or Frijoles viruses among 140 human sera tested (Berge 1975). No data is available on the role of Caimito or Nigue viruses as human pathogens.

The epidemiology of Panamanian PF group viruses is quite distinct from that of Old World PF group viruses. In the Old World, a close association exists between PF viruses and the insect vector, Phlebotomus papatasi. The distribution of these viruses, especially Naples and Sicilian viruses, closely follows the distribution of the vector (Tesh et al. 1976). In the New World, especially in Panama where considerable work has been done, many different PF viruses are found within a very limited geographical area, and several different species of sandflies appear to be involved in the maintenance of these viruses in nature (Tesh et al. 1974; Tesh et al. 1975).

Panamanian sandflies most commonly found infected include Lutzomyia trapidoi and L. ylephilator (Tesh et al. 1974). Virus has been recovered from both males and females of some species of New World sandflies, which indicates that transovarial transmission of PF group viruses probably occurs, and may provide one means of virus survival during conditions adverse to vertebrate mediated virus transmission (Tesh and Chaniotis 1975). Actual laboratory transmission studies using Lutzomyia and Panamanian PF group viruses have not been attempted, consequently the true vectorial capacity of these species has not been demonstrated.

The basic biology of New World sandflies has been investigated in some detail. Among the subject areas about which a reasonable amount of data are available are: Taxonomy (numerous papers by Hertig and Fairchild; Chaniotis 1974; Young 1979); host preferences (Thatcher 1968; Tesh et al. 1971; Tesh et al. 1972; WHO project currently in progress at GML); microclimatic requirements (Read 1977; Read et al. 1978); biology of immature sandflies (Thatcher 1968; Rutledge and Mosser 1971; Rutledge and Ellenwood 1974 a, b, c); behavior of adult sandflies (Cha-

niotis et al. 1971; Chaniotis et al. 1972; Chaniotis et al. 1974; Chaniotis and Correa 1974); and laboratory rearing of sandflies (Chaniotis 1974; 1975).

Unfortunately the establishment and maintenance of laboratory colonies of sandflies is a very difficult, tedious and time-consuming operation. While several significant questions best addressed through laboratory experimentation using colonized sandflies remain to be answered, the difficulty of maintaining such colonies continues to preclude this work. Laboratory transmission experiments might be one area of interest for future investigations following the initial phase of this project, should Panamanian PF viruses be shown to be important pathogens to high exposure populations.

Aside from the isolation of Chagres and Punta Toro viruses from man, no information is available on the role of vertebrates in the maintenance of Panamanian PF group viruses. In other regions of the world, mammals are the primary vertebrate hosts for PF group viruses, and it seems reasonable to assume that mammals are the most important feral hosts in Panama as well.

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Table 2. Phlebotomus fever group viruses and their ability to cause human illness.

<u>Virus</u>	<u>Clinical Disease</u>	<u>Antibody</u>	<u>No. Data</u>
SFN	+	+	
SFS	+	+	
KAR		+	
SAL		-	
AMT		+	
Sud An 754-61		+	
GOR		+	
SAF		+	
AGU		-	
CAC		+	
CAI			+
CHG	+	+	
FRI		-	
NIQ			+
PT	+	+	
ANH			+
BUJ		+	
CDU	+	+	
ICO		-	
ITP		+	
PAC		-	
URU			+
Co Ar 3319			+
RG		-	

B. Incidence and prevalence of Phlebotomus fever group viruses among American Military Personnel assigned to Panama.

OBJECTIVES: To determine the incidence and prevalence of Phlebotomus fever group viruses in American military personnel stationed in Panama.

BACKGROUND: Two Phlebotomus fever group viruses have been isolated from clinically ill military personnel following field exposure in Panama. Chagres (CHG) virus was first isolated from the blood of a febrile Nicaraguan officer while he participated in jungle warfare training at Fort Sherman (Peralta et al. 1965). Punta Toro (PT) virus was first isolated from the blood of a febrile American soldier, also following training in jungle warfare at Fort Sherman (Berge, 1970). Additional isolations of CHG virus have been made from clinically ill persons exposed in Panama (Srihongse and Johnson 1974). Additional isolations of PT virus from febrile patients have also been made, but these have not been reported in the scientific literature (Peralta, personal communications). To date, no attempt has been made to document either the incidence or prevalence of infection with Phlebotomus fever group viruses among populations with sustained field exposure in Panama. This information is crucial to an understanding of the impact of these viruses on the health of persons residing either temporarily or permanently in Panama.

Results presented in this section depict our attempts to determine the prevalence of antibody to Phlebotomus fever group viruses among American military personnel stationed in Panama.

DESCRIPTION: Four American military units are located in Panama which frequently engage in field training exercises. These are the 3rd Battalion, 5th Infantry stationed at Fort Kobbe, and the 4th Battalion, 20th Infantry at Fort Clayton, both of which are on the Pacific side of Panama. On the Atlantic side are the 4th Battalion, 10th Infantry stationed at Fort Davis, and the 3rd Battalion, 7th Special Forces Group stationed at Fort Gulick. Each battalion contains approximately 7-800 men, except the Special Forces Group at Fort Gulick, which has slightly over 300 men.

Within each battalion are 3 infantry companies (A, B, and C) a combat support company and a headquarters company. The infantry companies and the combat support company are engaged in training exercises or maneuvers which take them into the forest almost every week, thus they represent the segment with the highest rate of field exposure among U. S. military stationed in Panama. The headquarters companies also enter the field routinely, but not as frequently as the other companies. Several support units are located on each

post which have little or no field exposure. Consequently it will be possible to draw a sample of the US Military population such that a gradation exists in the degree of field exposure.

Personnel assigned to these units remain in Panama for various lengths of time. Generally an individual not accompanied by dependents has a tour of duty of 24 months. Those accompanied usually have a 36 month tour. It is not uncommon, however, to have people rotate out after as little as 12 months in the unit. Table 3 presents a summary of the forecasted personnel rotations between August 1979 and December 1981 for the 4 units under surveillance. It can be noted that approximately 83% of the current troop strength is forecasted to rotate during this period.

Various echelons of medical care are provided to personnel of these units. Each battalion is serviced by a battalion aid station, which is staffed by a warrant officer physicians assistant and a variety of medics. The battalion aid station is usually the first level of medical care provided to a sick or injured soldier; i.e. sick call is held there, etc. On the Atlantic side, the next level of treatment is provided by health clinic at Fort Gulick. Physicians staff this clinic, but patients are seen almost entirely on an out patient basis. Patients requiring hospitalization are transferred to Coco Solo Hospital. On the Pacific side, a health clinic is located at Fort Clayton which is similar to the one at Fort Gulick. Patients requiring hospitalization are handled at Gorgas Hospital.

Field training exercises are conducted in essentially undisturbed tropical forests which extend 5 miles inland on either side of the Panama Canal. Those companies which frequently engage in training exercises spend approximately 40% of their time, on an annual basis, in the field. Typically field training exercises last 3 days and 2 nights, but longer exercises are not uncommon.

An attempt will be made to determine the incidence and prevalence of Phlebotomus fever group viruses by documenting the acquisition of antibody to these viruses among the population under surveillance. Sera will be collected in two separate programs. First, we will attempt to collect sera from all members of each battalion as they are assigned to the unit and as they rotate out. Collecting sera as individuals rotate in and out will result in the least disruption to normal training activities, yet will eventually result in a complete pre and post-exposure serum sample for most individuals assigned. In addition, such a protocol will allow immediate tes-

(ting of post-exposure samples. While it will not be possible to document seroconversions from the initial post-exposure samples collected, since they will lack a pre-exposure serum sample, they will provide an early indicator of the prevalence of Phlebotomus fever group infection and may highlight geographical areas of greatest virus activity. This will allow early modification of the protocol, if necessary, or the establishment of a field program to investigate the exact source of infection.

The second source of sera for this study involves collecting blood samples at the time of drug screening. Army regulations (AR 600-85) require that a random sample of at least 60% of all military personnel 25 years old or younger be screened annually for drug abuse. All units under our surveillance program are actively participating in this drug screening program, and we anticipate that such screening will provide an excellent opportunity for us to draw serum samples from this segment of the population. These collections will provide us with a large cross-sectional sample of much of the population early on in the study.

Participation in this study will be on a strictly voluntary basis. Each participant will be informed of the risks involved, the objective of the study, and the benefits to be gained, both to the individual and to the Army. When blood collections are scheduled to coincide with drug screening, a representative of the project will be on hand to introduce the study and to answer questions. All volunteers will be asked to sign an informed consent statement, a copy of which is attached as Appendix 1. When single blood samples are drawn from individuals as they rotate in and out of the units under surveillance, the medic drawing the sample will explain the study and request that the informed consent statement be signed. He will attempt to answer any questions which relate to the study and will refer those he is unable to answer to the GML staff by phone or in person for response. The informed consent statement will have a concise summary of the project, its objectives and potential benefits, and the risks associated with venipuncture. Signed informed consent statements will be maintained at GML indefinitely.

PROGRESS: Formal request to conduct this study has been made to Headquarters, 193rd Infantry Brigade through Col. Lehardy, Deputy Commander Operations and Training, and approval has been granted. Formal approval has also been received from Col. Birriel-Carmona, M.D. Director of Health Services, US Southern Command. On 18 July 1979 Col. Birriel distributed a memorandum to the commanders of each unit to be followed requesting their support. A copy of that memorandum is en-

closed as Appendix 2 .

Contact has been made with the battalion aid station personnel who support each unit under study. Personnel at each station were eager to cooperate in this program and have agreed to draw blood samples for both the seroconversion study and the acute disease program. Early results indicate that good cooperation from these people can be expected.

A total of 1032 sera have been collected from military personnel assigned to the 4 units under surveillance. Results of preliminary screening tests using a 1:4 serum dilution and testing against Chagres (CHG) virus are available. These results are summarized on Tables 4-7. Sera were considered to be positive on screening if they neutralized \geq 90% of the plaque dose, and \pm if they neutralized 50-89%. Those neutralizing less than 50% were considered negative. Titrations of positive and \pm sera are in progress, but only limited results are now available.

At Fort Davis, a total of 484 sera have been collected and tested for antibody to CHG virus to date. Of these, 1 (0.2%) was considered positive, and 22 (4.5%) were considered \pm . The remaining 461 sera were negative. The single positive sera titrated 1:8 to CHG virus. (90% reduction at 1:8; 50% reduction at 1:32).

At Fort Clayton, 495 sera have been tested for antibody to CHG virus to date. Of these, 2 (0.4%) were positive and 28 (5.7%) were \pm . The 2 positive sera have been titrated and are 1:128 and 1:16 (90% reduction) against CHG virus. We have yet to test these sera against other Phlebotomus group viruses.

Only 49 sera have been collected from Fort Kobbe to date. All these sera have been tested for antibody to CHG virus, and 1 (2.0%) was \pm . No positives have been found.

Only 4 sera have been collected from the Special Forces Group at Fort Gulick, and all sera have been negative.

A limited number of sera have been screened for antibody to Punta Toro (PT) virus, also at a 1:4 dilution. All samples tested were from Fort Davis. Of the 158 sera tested, 3 (1.9%) were positive and none \pm . We have yet to titrate these positive sera; however, one of the 4 positives is the same individual who titrated 1:8 for CHG virus. These results are presented in Table 8. A review of health records of the positive individuals indicates that of the 3 persons antibody positive

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to CHG virus, 2 experienced febrile episodes since arriving in Panama which were severe enough for them to report to sick call. Of the 3 positive for PT virus, 2 had records of febrile episodes. In all instances a diagnosis of "flu syndrome" was made. It is impossible to determine, however, if these clinical episodes were actually the result of infection with a Phlebotomus fever group agent.

COMMENT: The preliminary results presented here indicate that US military personnel assigned in Panama are being infected with CHG and PT viruses. Of the 2, PT virus appears to be the more prevalent. The relatively large percent of sera which tested + to CHG virus may indicate cross-reactivity to another Phlebotomus fever group virus. This will become evident as testing is continued.

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Table 3. Forecast of anticipated troop rotations between August 1979 and December 1981 among units to be followed. Total strength estimates based on August 1979 figures.

Month	Year	Ft. Davis	Ft. Clayton	Ft. Kobbe	Ft. Gulick	Total
Aug.	1979	20	8	23	8	59
Sept.		14	15	12	6	47
Oct.		24	24	24	9	81
Nov.		27	18	30	8	83
Dec.		33	16	28	13	90
Jan.	1980	28	40	31	12	111
Feb.		12	27	24	2	65
Mar.		19	24	11	7	61
Apr.		34	15	17	8	74
May		36	17	22	13	88
June		24	33	33	18	108
July		33	27	29	9	98
Aug.		23	21	23	10	77
Sept.		20	26	14	11	71
Oct.		27	30	25	11	93
Nov.		28	35	25	8	96
Dec.		18	18	30	8	74
Jan.	1981	41	43	43	12	139
Feb.		25	22	19	5	71
Mar.		28	20	19	7	74
Apr.		25	20	25	11	81
May		19	36	35	10	100
June		23	38	40	2	103
July		16	13	17	7	53
Aug.		8	8	14	5	35
Sept.		4	15	11	2	32
Oct.		15	6	9	10	40
Nov.		5	13	4	1	23
Dec.		3	10	8	6	27
Total		632	638	645	239	2,154
Strenght		745	789	751	286	2,511
% Turnover		84 %	80 %	85 %	83 %	83.2 %

Table 4 . Sera collected from personnel of the 4th BTN, 10th INF, Fort Davis, and tested at 1:4 screening dilution against 20-50 plaque forming units of Chagres virus; August-September, 1979.

Company	Pos.	<u>±</u>	Neg.	Total tested
A		3 (4.6%)	61	64
B		2 (2.9%)	66	68
C		3 (3.8%)	76	79
HQ-D		1 (2%)	48	49
HHC		5 (6.2%)	81	86
Trans.		2 (4.7%)	41	43
CSC	1 (1.6%)	4 (6.3%)	58	63
Special Forces			5	5
Unk.		2 (7.4%)	25	27
Total	1 (0.2%)	22 (4.5%)	461 (95.3%)	484

Table 5. Sera collected from personnel of the 4th BTN, 20th INF, and associated units, Fort Clayton, and tested at a 1:4 screening dilution against 20-50 plaque forming units of Chagres virus; August-September, 1979.

Company	Pos.	<u>±</u>	Neg.	Total tested
A	2 (4.3%)	5 (10.6%)	40	47
B		2 (2.8%)	69	71
C		4 (5.1%)	74	78
CSC		8 (80%)	2	10
HHC		3 (2.0%)	150	153
475 th		1 (2.0%)	47	48
Trans.				
396 th Sig		1 (1.5%)	64	65
Unk.		4 (17.4%)	19	23
Total	2 (0.4%)	28 (5.7%)	465 (93.9%)	495

Table 6. Sera collected from personnel of the 3rd BTN, 5th INF and associated units, Fort Kobbe, and tested at a 1:4 screening dilution against 20-50 plaque forming units of Chagres virus; August- September, 1979.

Company	Pos.	\pm	Neg.	Total tested
A			2	2
B			4	4
C			2	2
CSC		1 (33.3%)	2	3
HHC			15	15
518			3	3
Trans				
Unk.			20	20
Total		1 (2.0%)	48	49

Table 7. Sera collected from personnel assigned to the 3rd
BTN, 7th Special Forces Group, Fort Gulick and
tested at a 1:4 screening dilution against 20-50
plaque forming units of Chagres virus; August-
September, 1979.

Company	Pos.	<u>±</u>	Neg.	Total tested
A				
B			1	1
C				
CSC				
HHC			2	2
Trans.				
Unk.			1	1
Total			4	4

Table 8 . Sera collected from personnel assigned to the 4th
BTN, 10th INF, Fort Davis, and tested at a 1:4
Screening dilution against 20-50 plaque forming
units of Punta Toro virus; August -September, 1979.

Company	Pos.	<u>±</u>	Neg.	Total tested
C			73	73
CSC	2 (3.3%)		58	60
HHC	1 (4.3%)		22	23
Unk.			2	2
Total	3 (1.9%)		155	158

C. Acute Illness Surveillance

OBJECTIVE: This aspect of the study will attempt to determine the incidence of clinical illness caused by Phlebotomus fever group viruses among US military personnel assigned to Panama.

BACKGROUND: Two Panamanian Phlebotomus fever group viruses, Chagres and Punta Toro, are known to cause clinical illness in man. Both viruses were originally isolated from clinically ill military personnel as they participated in jungle warfare training at Fort Sherman, Canal Zone. No attempt has yet been made, however, to determine the incidence of infection with these viruses among high exposure human populations. Likewise, no information is available to determine the ratio of apparent to inapparent infections with these viruses. This study will provide useful information which will allow us to answer these questions.

DESCRIPTION: The target population for this aspect of the study will be the 4 field units with high field exposure which were previously described. Contact will be made with individuals who staff the primary health care facilities which support the units under study, and they will be informed of our interests in virus disease epidemiology. They will be requested to draw acute blood samples from all potential cases of arbovirus diseases and briefed on the appropriate methods of collecting and handling samples for virus isolation attempts. For purposes of this study, a case will be defined as any person suffering from a fever of 100°F or greater for at least 24 hrs, without obvious signs or symptoms of respiratory illness or bacterial infection. By using a very broad case definition, we hope to not only collect samples from all cases of Phlebotomus fever, but also to monitor other arboviruses as well. Formal channels will be established to enable clinic staff to notify our representative that acute samples have been taken and to have the samples collected. Acute samples will be assayed in Vero cell culture and duplicated litters of suckling mice for the presence of virus. All virus isolations made will be identified, if possible. When appropriate, convalescent sera will be collected. Priority will be given to "Feed-back" of information to cooperating individuals and units. Regardless of whether or not acute samples were drawn, a representative of the project will visit each clinic at least weekly in an effort to maintain interest in the project among cooperating units.

PROGRESS: This aspect of the study is just commencing, consequently few acute samples have been collected or tested. Table 9 presents a summary of all clinical samples collected to date, principle signs and symptoms of the patient, the attending physicians assistant's diagnosis and the status of our attempts to isolate virus in Vero cell culture and suckling mice.

A single acute sample has yielded virus so far. This sample was collected from a soldier stationed at Fort Davis (Atlantic side of Panama). Blood was drawn on day 4 of illness, when the patient had a temperature of 102.4°F. The sample first caused visible cytopathetic effect in Vero cell culture on day 7 post-inoculation, and killed suckling mice inoculated intracerebrally. Harvested cell culture supernate and suckling mouse brain suspensions were both successfully passed. Viral stocks of 3rd passage level Vero cell culture supernate were made for virus identification. An aliquote of this stock was titrated in Vero cell culture grown in 96 well panels. Plaques formed were uniform in size, clear cut and distinct, and approximately 1-2mm in diameter. This virus stock contains approximately 2.0×10^6 pfu/1.0ml.

A plaque reduction neutralization test has been done in an attempt to identify the isolate. Grouping antisera against alphaviruses, flaviviruses, Group C arboviruses, California group arboviruses and mouse hyperimmune ascitic fluids (MHAF) to Panamanian Phlebotomus fever group viruses were tested, as were the patient's acute and convalescent sera. Results are presented in Table 10. The acute sera, all grouping reagents, and MHAF to Punta Toro, Caimito, Frijoles, Nique, Aguacate, Itaporanga and Icoaraci viruses all failed to neutralize the isolate. The convalescent serum drawn 21 days after acute serum and MHAF to Chagres virus both neutralized the isolate to a significant extent.

COMMENT: It is difficult to determine an exact titer of the convalescent serum, since the serum reduces both the number and size of plaques. If a 90% reduction point in number of plaques alone is used, then the titer is 1:32. If 80% is used, the titer is 1:64, and if 50% is used, the titer is $\geq 1:256$. Bartelloni and Tesh (1976) observed similar results with convalescent sera from patients experimentally infected with Sicilian type sandfly fever virus. They selected a criteria which considered plaque size as well as percent reduction for titrations. Using their criteria for positivity of $\geq 80\%$ reduction and a plaque size approximately equal to the unneutralized dose, the titer of convalescent sera is $\geq 1:256$.

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Table 9. Summary of acute blood samples collected and virus isolation attempts made from military personnel under surveillance in Panama, Aug.-Sept. 1979.

<u>Patient</u>	<u>Age</u>	<u>Location and Co.</u>	<u>Symptoms</u>	<u>Temp.</u>	<u>Clinical Dx.</u>	<u>Results/Final Dx</u>
T. B.	23	Ft. Davis, C	Chills, HA, Dizziness	102.4	Flu syndrome	Chagres virus
R. H.	19	Ft. Kobbe, CSC	Sore throat, malaise	102.4	Viral URI	Neg.
K. K.		Ft. Kobbe, CSC	Sore throat, malaise	104	Acute tonsillitis	pending
N. J.	20	Ft. Kobbe, B	HA, sore throat leg pain	101.3		meningitis

Table 10. Results of a plaque reduction neutralization test to identify a virus isolation made from a patient seen at Fort Davis, Canal Zone, Aug. 1979.

<u>Serum or MHAF*</u>	<u>Test titer</u>	<u>Homologous titer</u>
Acute	<1:8	-
Convalescent	≥1:256	-
Group A	<1:8	$10^{3.2} - 10^{4.9**}$
Group B	<1:8	Not done
Group C	<1:8	$10^{3.1} - 10^{3.7**}$
California Group	<1:8	Not done
Caimito	<1:8	1:2560
Frijoles	<1:8	1:640
Nique	<1:8	1:80
Aguacate	<1:8	-
Punta Toro	<1:8	1:960
Chagres	≥1:256	1:1280
Itaporanga	<1:8	1:320
Icoaraci	<1:8	1:128***

* Mouse hyperimmune ascitic fluid

** Neutralization index in suckling mice

*** Complement fixation test titer.

D. Serological Survey of Las Perlas Islands

OBJECTIVE: This survey attempts to determine the prevalence of antibody to Chagres and other Phlebotomus fever group viruses among residents of Las Perlas Islands.

BACKGROUND: Srihongse and Johnson (1974) conducted a serological survey of Panama using the hemagglutination inhibition (HI) test to determine the antibody prevalence rate to Chagres virus. Their results showed a prevalence rate of about 5% throughout Panama, with the exception of Las Perlas Islands, where the rate was nearly 20%. This finding prompted them to suggest that Chagres virus might be endemic in Las Perlas Islands.

The ecological characteristics of Las Perlas Islands make them an ideal area to investigate the endemic maintenance cycle of Chagres virus, if it is indeed endemic there. This is because the species diversity of the resident fauna is quite limited as compared to mainland Panama. For example less than 10 species of sandflies are known from Las Perlas Islands, while nearly 100 have been described from mainland Panama.

Prior to initiating field studies in Las Perlas, it is necessary to confirm the observations of Srihongse and Johnson and demonstrate that the virus is, in fact, endemic there.

DESCRIPTION: Blood samples were collected from residents of San Miguel on Isle del Rey and of Pedro Gonzalez on Isle Pedro Gonzalez on 30 and 31 May 1979. Blood samples were collected primarily from school children. Light traps were also operated in and around both cities in an attempt to collect sandflies. Collections were also made by mechanical aspirators around buttress trees in both localities.

Sera from Las Perlas Islands were tested by plaque reduction neutralization tests (PRNT) at a 1:4 dilution against 20-50 plaque forming units of Chagres virus on Vero cells grown in 96 well panels following procedures described earlier.

Sandflies collected in Las Perlas Islands were identified by Dr. Byron Chaniotis of Preventive Medicine Department, U. S. Army, Canal Zone.

PROGRESS: 280 blood samples were collected from residents of San Miguel and Pedro Gonzalez. Sera were collected primarily from school aged children, although some adults and younger children were also sampled. All sera have been screened for antibody to Chagres virus at a 1:4 serum dilu-

tion, and none were considered positive. Some sera were \pm , which may indicate antibody to a heterologous Phlebotomus group virus. Results are summarized in Table 11 .

A total of 235 sandflies of 6 species were collected from Las Perlas Island. Lutzomyia cayennensis and L. trinidadensis were the most abundant species collected. Two anthropophilic species were collected, L. gomezi and a single specimen of L. panamensis. Results of these collections are summarized in Table 12.

COMMENT: Results of our serological survey failed to document the endemicity of Chagres virus in Las Perlas Islands. It is possible that Chagres virus was endemic some time in the past, but is no longer prevalent, and our sample failed to include sufficient adults to document this since school-aged children were primarily sampled.

It is also possible that the HI test used by Srihongse and Johnson failed to differentiate antibody to Chagres virus from antibody to another heterologous Phlebotomus group virus, and that their results actually reflect the prevalence of one or more additional Phlebotomus fever group viruses. Tesh et al. (1975) demonstrated previously that the HI test is more cross-reactive than the PRNT. We plan to test all sera collected against each recognized Panamanian strains of Phlebotomus fever virus in an attempt to resolve this point.

Our preliminary results do not justify the establishment of investigations of the endemic maintenance cycle of Chagres virus in Las Perlas Islands. Consequently, we will continue to search for a suitable study site.

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Table 11. Summary of plaque reduction neutralization test results on 280 sera collected from 2 localities in the Perlas Islands, Panama, May, 1979, and tested at a 1:4 serum dilution against 20-50 plaque forming units of Chagres virus on Vero cells grown in culture.

Pedro Gonzalez

<u>Age</u>	<u>Pos*</u>	<u>+ *</u>	<u>Neg.*</u>	<u>Total</u>
<u><4</u>		1	1	2
5-6		1	10	11
7-8			15	15
9-10			18	18
11-12			18	18
13 or older			36	<u>36</u>
Total		2 (2%)	98	100

San Miguel

<u>≤ 4</u>		1	2	3
5-6			11	11
7-8			31	31
9-10			26	26
11-12		1	31	32
13 or older		3	74	77
Total		5 (2.8%)	175	180

* Pos= \geq 90% reduction; += 50-89% reduction; Neg= \leq 49% reduction.

Table 12. Phlebotomine sandflies collected in Las Perlas Islands, Panama, 30-31 May 1979.

<u>Species</u>	<u>Light Trap</u>	<u>Aspirated</u>
<u>San Miguel:</u>		
<u>Lutzomyia cayennensis</u>	60 ♂, 29 ♀	4 ♂, 3 ♀
<u>L. gomezi*</u>	6 ♂, 17 ♀	
<u>L. micropyga</u>		3 ♂
<u>L. panamensis*</u>	1 ♂	
<u>L. trinidadensis</u>	1 ♂ 4 ♀	28 ♂, 18 ♀
<u>Pedro Gonzalez:</u>		
<u>L. atroclavata</u>	1 ♂	3 ♂ 1 ♀
<u>L. micropyga</u>	2 ♂	2 ♂
<u>L. trinidadensis</u>	19 ♂ 22 ♀	7 ♂ 4 ♀

* anthropophilic species

Appendix 1a. Informed consent form, facing page.

**VOLUNTEER AGREEMENT
(Military Personnel)**

I, _____, having full capacity to consent, do hereby volunteer to participate in a research study entitled: Sandfly fever viruses among American military stationed Panama under the direction of MAJ James LeDuc.

The implications of my voluntary participations; the nature, duration and purpose; the methods and means by which it is to be conducted; and the inconveniences and hazards which may reasonably be expected have been explained to me by _____, and are set forth on the reverse side of this agreement, which I have initialed. I have been given an opportunity to ask questions concerning this investigational study, and any such questions have been answered to my full and complete satisfaction.

I understand that I may at any time during the course of this study revoke my consent, and withdraw from the study without prejudice; however, I may be required to undergo certain further examinations, if in the opinion of the attending physician, such examinations are necessary for my health or well being.

Signature

Date

I was present during the explanation referred to above, as well as the volunteer's opportunity for questions, and hereby witness his signature.

Witness' Signature

Date

Appendix 1b. Informed consent form, opposite page.

So you'll know

We are attempting to take blood samples from all military personnel assigned to field units in Panama. We hope to get a sample from you at the time you are assigned to your unit, another before you rotate out, and one or two during your tour here. These samples will be tested to see if you have developed immunity to certain tropical diseases while you were in Panama. The information we get from this study will help us to evaluate the need for development of new vaccines against these tropical diseases.

As a volunteer in this study, you must be aware of the risks involved. We will be taking a small amount of blood from you by venipuncture. This procedure will cause momentary discomfort, and occasionally will leave a bruise (hematoma) where the blood was drawn. The bruise will be painless and will disappear over a period of a few days. Very rarely an infection may result from venipuncture. If this happens, you will be treated by the staff at the health clinic. The number of blood samples drawn from you will depend on how long you stay in Panama; ideally we would like one sample every year.

We will test your blood at Gorgas Memorial Laboratory and will return the test results and an explanation of these results to you at your unit usually within a few months of collecting the sample.

Please initial the bottom of this page, and read and sign the volunteer agreement on the other side. If you have any additional questions regarding this study, please contact MAJ LeDuc or his staff at Gorgas Memorial Laboratory Bldg 265, Ancon, Telephone 52-5533/52-5544. We appreciate your cooperation in helping us complete this important study.

Thanks,

MAJ LeDuc
Gorgas Memorial Laboratory

Your initials

Appendix 2. Formal Request for Cooperation from Col. Birriel, M.D.,
Director of Health Services.

AFZU-ME-CO

18 July 1979

SUBJECT: Study of Tropical Diseases Among US Military Personnel

SEE DISTRIBUTION

1. A study of the incidence and prevalence of tropical diseases among US military personnel stationed in Panama will begin on or about 1 August 1979. The project will be conducted by the Gorgas Memorial Laboratory, with Major James LeDuc as the principal investigator. A brief summary of the objective of this study is attached for your information. The study has been approved by Headquarters, 193d Infantry Brigade (Canal Zone).
2. Your cooperation with Major LeDuc in conducting this study is encouraged.
3. For additional information, please contact Major LeDuc at Gorgas Memorial Laboratory, telephone numbers 52-5533 and 52-5544.

1 Incl
as

TOMAS BIRRIEL-CARMONA, M.D.
Colonel, MC
Director, Health Services

DISTRIBUTION

Deputy Commander (Ops & Tng)
Commander, 3d Bn, 5th Inf
Commander, 4th Bn, 20th Inf
Commander, 4th Bn, 10th Inf
Commander, 3d Bn, 7th SFG

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
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10 NO./CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62770A	3M762770A802	00	011			
b. CONTRIBUTING							
c. CONTRIBUTING	CARDS 114F						
11. TITLE (Precede with Security Classification Code) ^a							
(U) Health Care and Management of Laboratory Animals							
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010100 Microbiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
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17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER ^a				FISCAL		79	
c. TYPE:				YEAR		3.5	
d. AMOUNT:				CURRENCY		557	
e. KIND OF AWARD:				80		4.0	
f. CUM. AMT.				300			
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME ^a Walter Reed Army Institute of Research				NAME ^a Walter Reed Army Institute of Research			
ADDRESS ^a Washington, DC 20012				Division of Veterinary Medicine			
				ADDRESS ^a Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Russell, Philip K., COL, MC				NAME ^a Rogul, Marvin, PhD			
TELEPHONE: 202 576-3551				TELEPHONE: 202 576-3011			
				SOCIAL SECURITY ACCOUNT NUMBER			
22. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence not considered				NAME: Binn, Leonard N., PhD			
				NAME:			
23. KEYWORDS (Precede EACH with Security Classification Code) (U) Disease surveillance; (U) Klebsiella pneumoniae;							
(U) Aotus; (U) Guinea pig salmonellosis; (U) Laboratory dog viruses; (U) Cat viruses							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Furnish individual paragraphs identified by number precede text of each with Security Classification Code)							
<p>23. (U) To investigate diseases and/or conditions affecting laboratory animals used specifically for military research to enhance production quality and health management and to provide research animals free of known or potential pathogens. The ability to provide clinical diagnosis to laboratory animal problems peculiar to the WRAIR animal colonies is critical to the specific research conducted by the WRAIR. The establishment of a disease data storage/retrieval system will provide unique epizootiological information not available from any other laboratory source.</p> <p>24. (U) Conventional epidemiologic, pathologic and microbiologic methods are employed; unconventional procedures are developed as needed.</p> <p>25. (U) 78 10 - 77 09 Bacteriological and animal data was stored in the computer for future use. Resistance transfer factors of Klebsiella pneumoniae have been passed to coliforms and are now being molecularly characterized. New broth formulation was found to be more selective and sensitive than any one agar medium for the isolation of K pneumoniae. Antibiotic feeding trials were started for evaluation in treatment of guinea pig salmonellosis. A protein antigen has been extracted from Salmonella typhimurium for testing past or present guinea pig infections.</p> <p>Antigenic diversity was found in cytomegalic and adenoviruses isolated from Aotus monkeys. Feline panleukopenia-like and corona viruses were isolated from government owned dogs during diarrhea epizootics. Rhinotracheitis and calciviruses were isolated from sick cats at the WRAIR colony. Serological evidence indicated infections with feline infectious peritonitis or related viruses. High kitten mortality was associated with thymic lymphocytic atrophy. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.</p>							

^a Available to contractors upon originator's approval

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Project 3M762770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 011 Health Care and Management of Laboratory Animals

Investigators:

Principal: M. Rogul, PhD and L. N. Binn, PhD
Associates: CPT D. G. Martin, VC; R. L. Marchwicki, BS;
CPT S. R. Lamb III, VC; J. Brendle, BS; K. F.
Noon, M Sc; R. E. Sims, BS; SP5 R. E. Thomas, BS;
SP4 M. P. Riley; SP4 R. A. Von der Porten; SP4
L. D. Palmer; SP4 R. Soltero; SP5 E. Wolff,
CPT L. A. McKinney, VC; CPT C. Keenan, VC;
P. Gemski, PhD; and MAJ R. M. Bunte, VC

Description:

Investigate diseases and/or conditions affecting laboratory animals used specifically for military research to enhance production, quality and health management and to provide research animals free of known potential pathogens.

During the reporting period activities have included: (1) investigation of microbiological aspects, bacterial and viral, of morbidity and mortality among animal colonies at the WRAIR, a two year comparison (2) characterization of Klebsiella pneumoniae antibiotic resistance (3) isolation techniques for K pneumoniae (4) definition of Aotus monkey viruses and (5) epizootiology of laboratory cat diseases (6) studies of canine viruses in diarrheal disease.

Progress:

1. Diagnosis of disease, surveillance and quality control of laboratory animals; a two year comparison.

The criteria and designs for an animal disease surveillance program at the Walter Reed Army Institute of Research and related facilities were described (1). The program has continued and has encompassed 4 major areas: (1) identification of pathogens isolated from sick or moribund animals, (2) routine survey of the rodent production colonies to include viral serology, (3) special surveys for any animal disease problems, and (4) reduction of the data to a form that is compatible with data processing.

Progress September 1977 through August 1978: In addition to epizootiological studies of disease problems among owl monkeys in the WRAIR colony, specimens were received from sick or moribund monkeys for qualitative aerobic and anaerobic bacteriologic examination. Specimens were examined from approximately 76 sick Aotus monkeys. Klebsiella pneumoniae was isolated from throats, feces, vaginas, wounds, or abscesses of 18 of these monkeys. Staphylococcus aureus was the second most frequent pathogen isolated (15 times) and sometimes was found in conjunction with K pneumoniae. The most common sources of S aureus was abscesses, wounds, eyes, or throats. Pseudomonas aeruginosa was isolated thrice and Streptococcus pyogenes once. Listeria monocytogenes was recovered from 1 aborted fetus, and the organism also was isolated from a vaginal specimen from the mother.

Specimens were examined for 46 owl monkeys that died during the report period. As expected, K pneumoniae was the principal bacterium recovered. This organism was isolated from 24 of the monkeys, mainly from tissues that usually are sterile. All typable isolates were biotype indole negative, serotype 2. Two monkeys yielded untypable serotypes which were biotype indole positive. Six of the monkeys had died during a nephrology study. Pseudomonas maltophilia, an opportunist, was cultured from 4 of these monkeys after necropsy. Fourteen owl monkeys yielded single isolations of Gram negative enteric bacilli from necropsy tissues. Other bacteria isolated included L monocytogenes and a few other bacteria which did not form a specific class. Eight of the 46 dead monkeys had mixed bacterial infections. By contrast, the necropsy tissues examined for an additional 8 monkeys were bacteriologically sterile.

Respiratory disease, and occasionally a case of intestinal disease, continues to be the principal cause of morbidity and mortality among the other subhuman primate species. Specimens were received from 14 sick baboons. The major pathogens found were S aureus in 4, Pasteurella multocida in 3, and Shigella flexneri in 1. Three baboons died, 2 from mixed bacterial infections and 1 from S flexneri. Twenty rhesus monkeys became sick and 8 died during the year. Four of the rhesus were included in a study that required the use of indwelling catheters. Each of these had developed P aeruginosa and S aureus infections. For the 8 rhesus monkeys that died, there was no significant bacterium common to the deaths. Ten cynomolgus monkeys died; however, no major or common pathogens were isolated.

Diagnostic specimens were received from dogs, cats, and rabbits for bacteriologic examination. Most of the specimens from 17 sick and 8 dead dogs were received in conjunction with viral respiratory disease. No specific bacterial pathogens were noted, except for 5 isolations of Bordetella bronchiseptica. Pasteurella multocida was isolated from 3 sick cats and B bronchiseptica from an additional 3. Otherwise, no principal pathogens were isolated from the specimens submitted from 28 sick and 5 dead cats, most with respiratory disease and/or conjunctivitis. Specimens from 46 rabbits were examined. The feces of many of these rabbits contained large numbers of Gram negative rods, especially K pneumoniae and other Enterobacteriaceae. The majority of the samples other than feces were swab specimens of the nasal cavity and skin lesions. Pasteurella multocida 4 isolates and B bronchiseptica 4 isolates were the significant organisms isolated from these specimens. An additional group of specimens was received from 52 moribund rabbits. The major bacterial pathogens recovered from necropsy tissues were P multocida 15 isolates, K pneumoniae 9 isolates, P aeruginosa 10 isolates, and E coli 8 isolates. Some of the dead rabbits had been housed in a USAIDR facility where rabbits, rats, mice, and guinea pigs were maintained in the same room. The close proximity resulted in cross infection among the different species. Gentamycin was determined to be the antibiotic of choice for treating all the bacterial and mycoplasma pathogens detected.

The routine surveillance and quality control program continued to detect those pathogens or opportunists most likely to be encountered diagnostically. On 30 January 1978, Salmonella typhimurium var. Copenhagen (Group B serotype) was detected during the routine survey of the WRAIR guinea pig colony. Detection of the pathogen occurred prior to the onset of overt signs of illness. This organism appeared to be highly infectious, but of low virulence, for guinea pigs. The major signs of disease, when expressed, were weight loss, slight alopecia, and unthriftiness. Premature deaths due to salmonellosis were not observed. The isolate of S typhimurium was sensitive to neomycin, chloramphenicol, gentamycin, and ampicillin, and was of intermediate sensitivity to tetracycline and streptomycin. Since some forms of salmonellosis are refractory to in vivo antibiotic therapy, an enhanced system of culling also was instituted in conjunction with increased bacteriologic surveillance. Examination of pooled fecal samples from each cage showed that the outbreaks occurred sporadically from one cage to another in each of the 2 guinea pig rooms. Cultures positive for S typhimurium were detected each month from

February through July. All fecal specimens tested in August were negative. Continued surveillance testing will verify whether eradication of the enzootic foci has been attained. In May and August 1977, a malonate negative biotype of K pneumoniae (or possibly K ozaenae) was isolated from intestinal and stomach abscesses of each of 2 rats during the routine survey of the production colony. Similar abscesses caused by a malonate negative strain of K pneumoniae were detected in the intrapelvic region of a rat provided in November 1977 to the Department of Gastroenterology, Division of Medicine. At that time it was not anticipated that these findings would portend an epizootic of Klebsiella infection in the rat colony. However, in August 1978 such an epizootic commenced. A total of 23 infected rats have been observed. Each infected rat exhibited large abscesses of the ventral cervical region, commonly affecting the cervical lymph nodes. Occasionally there also was involvement of the inguinal lymph nodes. Klebsiella pneumoniae, serotype 5, was isolated from both groups of lymph nodes, as well as from the lungs, liver, spleen, and kidneys of affected rats. Antibiotic sensitivity tests indicated the causative agent was sensitive to gentamycin, tetracycline, kanamycin, amakacin, chloramphenicol, sulfa, and neomycin, but was resistant to lincomycin and carbenicillin. Further investigations have been initiated to define this disease syndrome and to establish definitive corrective measures, especially since Klebsiella sp have not been associated with naturally transmissible diseases of rats. During the report period, detectable disease problems caused by bacteria were not observed among the mouse colonies.

Overt diseases caused by viruses were not observed among the animals of the WRAIR mouse, rat, and guinea pig colonies. The presence of select viral infections among these animals, however, was detected by routine seroassay surveillance of the colonies. The rats commonly had moderately high and prevalent antibody titers to PVM, Kilham rat virus, rat coronavirus, and Sendai virus, but were seronegative for LCM virus antibodies. Guinea pigs, likewise, were infected with and had moderate antibody titers to PVM. Infection of guinea pigs with SV5 and Sendai viruses was sporadic. Seroassay results for August through October 1977 showed a significant frequency of infection among the 3 mouse colonies with PVM, Sendai virus, and MHV (Table 1). A decision was made in December 1977 to terminate 1 of the mouse colonies because of the prevalence of these viral infections, especially MHV, and the potential deleterious effects the agents could have on research projects. A new mouse colony was established in

Room 3 of Bldg 512 in January 1978 using mice obtained from the Portage Colony of Charles River. Mice from the new colony were seronegative upon arrival for antibodies to each of the select viruses. Infections with the almost ubiquitous Sendai virus, however, developed among mice of the new colony within the first 90 days after being established. Within 180 days, mice seropositive for PVM and MHV were identified. Thus, the existing barrier system did not prevent cross-contamination with and concomitant infection of mice in the new colony. The relative rapidity with which the 3 viruses penetrated the barrier system primarily was attributed to deficiencies in design and construction of Bldg 512.

A data storage and retrieval system was developed as an adjunct to the surveillance and quality control program. The data base contained a capacity for bacteriologic and mycoplasma results, and also had a limited capacity for viral and mycologic findings. During the report period, programs were written for a data submission sheet and work sheets for aerobic and anaerobic bacteria and mycoplasma studies. These programs currently are in the first generation of editing and development.

September 1978 through August 1979: During the reporting period 1 September 1978 to 31 August 1979 the WRAIR Aotus colony numbered between 200 to 250 monkeys in Bldg 511. Specimens were bacteriologically examined in 15 instances (compared to 76 instances the previous reporting period for a similar sized colony). Staphylococcus aureus was the most frequent pathogen isolated (6 times) from eye and throat specimens. Pseudomonas aeruginosa was isolated three times and Klebsiella pneumoniae only once compared to 18 times the previous fiscal year.

Specimens were examined from 33 owl monkeys that died during this period. K pneumoniae was only isolated from two monkeys of the 33 (6%) compared to 24 of 46 dead (52%) the previous year. Streptococcus viridans, S aureus and Enteric organisms consisting of Proteus species and Escherichia coli were the bacteria predominantly associated with monkey deaths.

Twelve screening surveys were conducted for K pneumoniae. No solid conclusions were derived from the data except that isolations appeared to be highest after monkeys were fed oranges. The most striking finding was the reduction in the isolation of K pneumoniae from necropsy specimens. The reason for this sudden change is unknown. It may be that most of the suscep-

tible monkeys have died, or since the ventilation in Rooms 202J and K seemed to have improved in the last year, as evidenced by lower levels of NH_3 in these rooms and the quicker response of maintenance crews to complaints of air handler break downs, it may be, in part, that this K pneumoniae reduction was a response to the better air quality in the building.

From the other subhuman primates, 23 sick Cynomolgus (M. fascicularis) occasions were logged in the bacteriological records. S aureus was the most often isolated bacterium, usually from nose and throat cultures (9 times, possibly 12). Three isolations were probably suppressed because of successful antibiotic therapy. One case of Shigella flexneri was found. Ten illnesses could not be attributed to bacterial causes. Only two of these monkeys died during this period.

In the Rhesus monkey colony (M. mulatta) only 11 of 100-150 were examined for bacterial disease. S aureus was found on two occasions. The isolations of S aureus in the Rhesus, Cynomolgus and Owl monkey colonies coincided with the finding of S aureus on throat cultures from the animal technicians and caretakers in these areas. Seven necropsied Rhesus monkeys were examined. Bacterial diseases did not seem to be a major factor in the deaths of Rhesus monkeys.

Diagnostic specimens were received from dogs, cats and rabbits for bacteriological examination. Thirty-four instances of dog illnesses were recorded, ten of these were animals under experimentation. Some of these dogs (5 or 7) were new arrivals that were suffering from distemper. The latter dogs suffered and died with a variety of concomitant bacterial illnesses, including septicemic Clostridium perfringes. S aureus, E coli and Beta-hemolytic streptococci were the most frequent isolates from sick animals. Three of the experimental dogs were infected with Aeromonas hydrophila. Twenty-one necropsied dogs were examined for bacterial pathogens (5-7 deaths were probably distemper associated). Beta-hemolytic Streptococcal bacteria (4 times) and E coli (5 times) were the most often isolated organisms, but neither appeared to be a significant pathogen.

At least eleven cases of sick cats occurred. Seven of these cases were in September and October. Beta-hemolytic streptococci were isolated from the nasal passages of 4 animals, Bordetella bronchiseptica from two, P multocida from 1 cat, and Mycoplasma sp from the eyes of two cats. Only six cats

were necropsied during the year. No bacterium was isolated more than once from these six cats. A special investigation of cats in a neuropsychiatry study, showed that the cats were performing poorly because Enterobacter cloacae and P aeruginosa (and their concomitant metabolites) were in the saline solutions used in the experiments.

Sixteen dead rabbits were examined. Seven of the deaths were attributed to P multocida.

In the WRAIR rodent breeding colonies there did not appear to be any infectious disease problem in the mouse colony as no specimens were sent to the bacteriology laboratory during the year.

The specimens received from the guinea pig colony did not reflect the chronic salmonellosis previously found in this colony. Of the 1 sick and 10 dead guinea pigs examined, none yielded positive salmonella cultures. This was probably due to the effective testing and culling system. Within the last three months there was no S typhimurium isolated on 4 occasions which cultured 598 drawers of pooled feces. However, there is always the danger that these organisms were sequestered in the liver and spleen and not being shed in the feces with great frequency. Therefore, research is now in progress to treat these guinea pigs with effective antibiotics and devise ocular or dermal delayed hypersensitive tests which will demonstrate past or present infections.

Culling rats with obvious ventral cervical abscesses has not completely eradicated the disease caused by K pneumoniae serotype 5. Five sick rats during the year and one dead rat were infected principally with this organism. Twenty-four dead rats obtained from necropsy were bacteriologically examined. Three apparently died because of K pneumoniae infection, but 8 of the dead animals were infected with Corynebacterium kutscheri and this seemed to be the main cause of death. Pasteurella pneumotropica was isolated from only one rat in September 1978 and not since. Twelve experimental animals were investigated in a special survey. Two dead animals had C kutscheri isolated from their lungs, E coli from 2 lungs, and K pneumoniae and P pneumotropica from one lung each.

Surveillance of the WRAIR rodent colonies: The routine microbial survey of the guinea pig, rat and mice colonies did not detect any mycoplasmas in the rat or mice colonies. In March

and July 1979, *Pasteurella* species (probably *P. pneumotropica*) were isolated from two apparently healthy guinea pigs. In September 1978 and March 1979 the organism was isolated from two rats and *P. pneumotropica* was isolated from 9 mice from February to June 1979. The presence of this organism and other bacterial opportunists such as *C. kutscheri* are undesirable as they are known to proliferate and debilitate animals that are stressed by mycoplasma, bacteria, viruses and experimentation.

Viral seroassays indicated that the mice were still infected with Sendai, PVM and MHV viruses. The rats were seropositive for PVM, Kilham rat virus, Sendai and to some degree with Rat corona virus. The guinea pigs had antibodies to PVM, SV5 and Sendai viruses. None of the rodents had antibody to LCM virus.

Data from morbidity and mortality reports is now being entered and stored in the WRAIR computer.

Bacteriology support was provided to other government institutions during the year. Specimens from 5 monkeys, 6 dogs, 8 cats, 107 rabbits and a number of exotic laboratory animals were cultured and processed.

2. Selective isolation methods for low numbers of *Klebsiella pneumoniae*.

The occurrence of *Klebsiella pneumoniae* pathogens in the Aotus monkey and Wistar rat colonies requires that a constant monitoring and treatment scheme be available. Except for the general selective and enrichment enteric media, there was until recently no specific scheme for *K. pneumoniae*. Recently, Bagley and Seidler (2) devised a MacConkey agar medium containing inositol and carbenicillin (IC agar) specifically for the selection and identification of *K. pneumoniae*. Since far fewer Aotus deaths were being associated with *K. pneumoniae* during the last year and culling practices had reduced the number of the most obviously infected rats, it became necessary to culture large numbers of specimens with probably lower numbers of *K. pneumoniae*. The monitoring required a rapid, sensitive and highly selective medium to detect *K. pneumoniae*. To this end a broth variation of Seidler's agar medium was devised and tested.

The enrichment broth had the following composition: Bacto-peptone, 20 gm; Bacto-inositol, 10 gm; Bacto-oxgall, 5 gm; 60%

stock neutral red dye, 0.14 gm; and geopen carbenicillin activity = 900 u/mg) 0.07 gm in one liter water and adjusted to pH 7.3 (IC broth). Specimens consisting of swabs or feces were each inoculated directly to MacConkey agar, Brilliant green agar (BGA) and IC agar (direct methods). This was then compared to inoculation of IC broth procedures. The IC broth was observed for 3 days. Any broth which indicated acid production due to inositol fermentation was immediately inoculated on to MacConkey agar. The results of both throat and rectal isolates were found to be close when the direct methods (67% positive isolation) were compared to the enrichment method (71% K pneumoniae) isolation. However, the direct agar methods required 648 plates of agar to be processed (216 specimens) whereas only 48 of the 216 IC broths were inoculated to 48 MacConkey agar plates. Since the broths were selectively enriched the plates did not require searching for K pneumoniae colonies. Most of the strains isolated and tested, agglutinated in all of the K pneumoniae antisera pools. Of those which were typable, the majority were capsular types 2 and 68. There were occasions when fecal isolates appeared to be found in one method and not the other, but this is being further investigated because the direct agar isolates may have been Enterobacter species as well as Klebsiella sp. The results were Klebsiella-Enterobacter (K-E) found exclusively on the three direct agar isolation were 12 of 216 specimens (6%), exclusively in enrichment method 14 of 216 (7%) whereas 35 of 216 were in complete agreement in both methods. The greatest discrepancies were found when the individual agars were compared to the broth methods. One hundred twenty three (123) fecal samples were examined. From the broth method 21 K pneumoniae verified isolates were identified, compared to 14 K-E isolates from MacConkey agar, 13 from BGA and 8 from IC agar. It was obvious that the enrichment method was better able to isolate K pneumoniae than any direct agar medium alone.

3. Search for resistance transfer factors in antibiotic resistant Klebsiella pneumoniae isolates from Aotus monkeys.

The most frequent bacterium associated with Aotus trivirgatus deaths is Klebsiella pneumoniae capsule type 2 and indole negative. Generally, these and the untypable indole positive isolates were antibiotic sensitive. On two occasions indole +, untypable K pneumoniae isolated from two dead Aotus monkeys were found to be multiply resistant to antibiotics. We have also isolated non-type 2, indole negative strains which were multiply resistant. All of the environmental isolates tested

were sensitive to all the antibiotics tested except ampicillin. Most of these results were recorded in the WRAIR Annual Report 1977-1978.

The possibility exists that the resistances may in part have been due to plasmids and that these plasmids are transmissible among K pneumoniae strains and other species of Enterobacteriaceae.

Four antibiotic resistant aotus isolates were chosen for this study. They are listed and described in Table 2.

The original four resistant cultures were also treated with ethidium bromide to cure the clones of plasmids and increase the number of recipient antibiotic sensitive and indole negative clones. None of the 216 clones tested reverted from indole + to indole - and fewer antibiotic sensitive clones were found as compared to those which spontaneously reverted to sensitivity.

It is possible that too few clones were tested, that once cured the cells were immediately reinfected or that the ethidium bromide did not penetrate the cells. Controls using other Enterobacteriaceae with plasmids were cured during these experiments.

Attempts were made to show that the loss of antibiotic resistance coincided with a plasmid loss also. When cleared lysate methods were used on strains 120 and 27 the organisms failed to lyse. Another method which used a lysis salt precipitation (3) to obtain plasmid DNA, did work for an ethidium bromide treated strain of 27. The original strain was resistant to penicillin (P), erythromycin (E), chloramphenicol (C), streptomycin (S) and tetracycline (T) and had two plasmid DNA. The ethidium bromide treated strain was sensitive to C, T and S and only had one plasmid DNA. Thus indicating that in this strain C, T and S resistance were linked on one plasmid whereas P and E were probably on another. In order to demonstrate any transmissibility, relatedness or uniqueness among these plasmids, mating experiments were devised.

Nitrosoguanidine was used to mutagenize original strains 120 and 27. It was hoped that this would produce nutritional auxotrophs which could donate resistance plasmids to plasmid cured K pneumoniae. The donors would then be inhibited in subsequent experiments by growth on deficient media which contained the proper antibiotics. Auxotrophic strains were

obtained by this method. Donor auxotrophs and recipient nutritional prototrophs were grown together in broth media for mating purposes. The mixture was then grown on nutritional and antibiotic selective media. It appeared that there was some kind of cross feeding and cross protection because both donor and recipient strains grew out on the selective media even though subsequent tests showed that the growth was not due to hybridized conjugates.

Donor-recipient crosses were attempted between the *Klebsiella* strain donors and either *Shigella flexneri* 24570 or *Escherichia coli* C 600 recipients. A dilution plating method was used. Recipient cells were to be chosen by either picking non-lactose fermenting colonies on inhibiting media or obvious *E coli* colonies. Unfortunately, the *K pneumoniae* cells also transferred lactose fermentation and did not seem to transfer resistance to either of these *E coli* or *Sh flexneri* strains. Another *E coli*, but the same *Sh flexneri* strains were used as recipients in a different mating method, but as of this date only one transconjugate has been obtained by an intensive plating method; *K pneumoniae* 27g (P, C, E, S, T) $P^S X$ *Sh flexneri* (sensitive) \rightarrow *Sh flexneri* (P, C, S, T) P^S . Lysates have not yet been made from this recipient.

E coli 3951 $P^{+/-} C^S E^S / r^S T^S N^r$ has been found to be a successful recipient when crossed with the *K pneumoniae* strains and *Shigella* transconjugants. The streptomycin and nalidixic resistance of this strain is chromosome and not plasmid. The results are listed in Table 3. It is now obvious that all four of the selected *K pneumoniae* isolates are capable of transferring antibiotic resistance among species in the Enterobacteriaceae. It remains to be seen if these resistances are related by plasmids and plasmid gene nucleotide sequences and can be transmitted to capsule type 2 *K pneumoniae* pathogens.

4. Virus studies of adult and infant Aotus monkeys.

Studies on the isolation and identification of viruses from Aotus monkeys were reported (4). With the exception of a single Herpes virus simplex isolation made at the onset of this study, the isolates have been either cytomegalo-like viruses (CMLV) or adenoviruses. During the past year, (1) viral isolation studies have continued primarily on the post-mortem tissues of adult and infant monkeys, (2) immunologic studies were begun to determine the serologic relationships among the CMLV and adenovirus isolates, (3) serologic surveys have detected measles virus and human hepatitis A virus antibodies in serums from Aotus monkeys. This report summarizes and evaluates the newer findings.

The CMLV were recovered from the salivary glands and throat specimens at essentially the same frequency as previously reported (4). Approximately 50% of the salivary glands examined contained CMLV (Table 4). Adenoviruses were recovered from the rectal swabs of 1 adult and 1 infant monkey. The remaining organs examined were free of virus. Further studies were made to determine the persistence of CMLV in throat secretions of Aotus monkeys. Two years after the first isolation, 4 of 5 monkeys had CMLV in their oral secretions. These findings, together with similar observations made previously at 1, 2, 3 and 12 months (4), suggests that many Aotus monkeys have a long lasting persistent CMLV infection. Attempts to recover CMLV from leukocytes or urine of these persistently infected monkeys were not successful.

Studies of the antigenic relations among CMLV isolates were initiated during the past year. In addition to the reference 7933T virus, two other isolates were examined. They were selected on the basis of homologous neutralization with little or no neutralization of the reference virus. Each of these two isolates produced typical CMVL cytopathic effects in cell cultures and was not neutralized by reference antisera against other human and simian herpesviruses. The isolates were purified by 3 successive terminal dilutions and seed virus pools prepared. Immune sera against these 3 CMLV were prepared in rabbits with serum-free virus pools. Results of microtiter serum-neutralization tests indicated that each isolate was antigenically distinct (Table 5). The only cross reaction detected was a minor one-way cross reaction between the reference 7933T and 43T isolate. Further studies are required to identify and classify the remaining CMLV isolates. Additional work will be required to develop tests for common CMLV antigens and antibodies. Such tests are needed to rapidly identify isolates, detect viral antigens, antibodies and their complexes in the tissues of Aotus monkeys.

Antigenic studies of adenovirus isolates from the WRAIR Aotus monkey colony also were carried out. Each of 4 isolates was purified by 3 successive terminal dilutions and virus pools prepared. Hyper-immune antiserums to these viruses were made in rabbits with serum-free virus preparations. During these studies, Shroyer et al (5) reported the recovery of 3 adenovirus types and 2 untyped isolates from Aotus monkeys at Letterman Army Institute of Research (LAIR). Two of the LAIR adenoviruses were related to other simian adenoviruses. The LAIR Type I virus was neutralized by squirrel monkey adenovirus Type I antiserum and LAIR Type II by rhesus monkey SV11 adenovirus antiserum. Antiserum to the LAIR adenoviruses was provided by Dr. Shroyer. Antisera to other simian adenoviruses was provided by Dr. Lester of South West Foundation for Medical Research, San Antonio, Texas. Microtiter serum neutralization tests were done to determine the antigenic relationships of the WRAIR Aotus adenoviruses to each other and to the LAIR adenoviruses. Cross serum neutralization tests of the WRAIR isolates revealed significant differences among all the isolates (Table 6). However, one-way cross reactions were observed between several pairs and in 2 instances, adenoviruses, 8138R-WR122R, and WR322R-WR122R, the difference in titers were significant in both directions. The findings suggested that there are several Aotus adenovirus types. Initial comparisons of the WRAIR isolates with antiserums against the LAIR and related adenovirus supported these observations. (Table 7). None of the WRAIR isolates were significantly neutralized by the LAIR Types I or II or related simian adenovirus antiserums. The WR32R isolate was partially neutralized by LAIR III antiserum. However, two of the remaining 3 isolates, were neutralized at high titer by one of the untyped LAIR adenovirus antiserums. The remaining WRAIR isolate 8138R, did not react significantly to any of the LAIR antiserums.

Two of the isolates are antigenically related to an untyped adenovirus from LAIR and another isolate partially related to the LAIR Type III. The findings made to date suggest that there are multiple Aotus adenovirus types and no one type has been recovered more frequently from the WRAIR Aotus colony. Further studies are required to clarify these antigenic relationships.

In addition to the virus isolation studies, serologic tests were done to detect measles and hepatitis A viruses antibodies in the WRAIR Aotus monkeys. These viruses have been associated with significant disease problems in other species of sub-human primates (6,7,8) but their importance for Aotus monkeys has not been determined. Although the WRAIR Aotus monkeys are housed in separate

rooms, potential exposure to these agents may occur from either other monkeys in the same building or from laboratory workers. Hepatitis A virus, also, is a major cause of disease in military populations and there is a critical need for laboratory animals to study this disease (9).

During the past year serum specimens have been obtained from nearly half the WRAIR Aotus colony. Approximately 75% (84 of 115) of the specimens were obtained from monkeys originating from other institutes or licensed vendors and the remaining serums from animals born at the WRAIR. The latter had not been used for experimental purposes and were 8 or more months-old. In contrast, many of the procured monkeys had been used for malaria research, cleared of their infections and shipped to WRAIR for breeding studies.

Examination of the serum specimens of these monkeys, revealed that 17 of 84 (20.3%) of the procured monkeys had hemagglutination-inhibition (HI) antibody to measles virus. With 2 exceptions the HI titers ranged from 1:4 to 1:32. (Table 8) The exceptional serums both titered 1:128. In contrast, only 1 of 31 (3.2%) WRAIR bred monkeys was seropositive and only at a 1:4 dilution. Subsequent tests on this serum confirmed these results, but a 21 day later serum specimen from this monkey had a HI titer of 1:512 or greater. The findings indicated that this monkey had a recent measles infection. Examination of earlier bleedings of the other measles seropositive monkeys, provided evidenced that 2 additional monkeys also were infected at the WRAIR. They were the 2 monkeys with high HI titers described above. None of these monkeys had any signs of measles. All 3 measles infections occurred in one room of monkeys and 2 of the infected monkeys were in the same cage. Subsequent bleedings over the next 2 months of the 55 sero-susceptible monkeys in this room did not reveal any other measles infections. During this period 5 monkeys died and histopathologic evidence of measles virus infection was not observed. Post-mortem tissues of 3 of these monkeys also were examined for measles virus without success. The findings indicate that measles virus infections occur in Aotus monkeys but the infection is not readily communicable. However, the pathogenicity of the virus(es) causing these infections are unknown and other strains may be more virulent. Therefore, continued precautions should be maintained to prevent exposure of Aotus monkeys to other species of sub-human primates. Pilot studies on the safety and communicability of attenuated measles virus vaccine in Aotus monkeys should be considered to prevent wild virus infections. The latter viruses may be more virulent in pregnant, newborn, or experimental animals.

In conjunction with the Department of Virus Diseases, WRAIR, hepatitis A antibody determinations (HAVAB Test, Abbott Laboratories) have been made on serum specimens from both procured and bred Aotus monkeys of the WRAIR colony. In addition, serum specimens were examined from 25 newly captured Panamanian Aotus monkeys. The latter specimens were obtained from Dr. J. Harper of the Gorgas Memorial Laboratory in the Canal Zone. Hepatitis A virus antibody reactivity was found in more than half the WRAIR procured monkeys, but rarely in WRAIR bred or newly captured Panamanian monkeys (Table 9).

The findings suggest that Aotus monkeys are susceptible to Hepatitis A virus infection. However, further infectivity studies of this virus in Aotus monkeys are required to confirm these observations. The limited number of animal hosts, i.e., chimpanzees and marmosets, for hepatitis A virus studies clearly indicates a critical need for additional laboratory animals. Studies of the pathogenicity of hepatitis A virus in Aotus monkeys are planned to evaluate the potential use of this monkey in hepatitis A virus research.

5. Studies of viral enteritis in laboratory dogs.

During 1978 epizootics of viral diarrhea occurred in pet and laboratory dogs in the United States (10), Canada (11), Great Britain (12), and Australia (13). Initially, from February to June 1978, coronaviruses were associated with these disease outbreaks. Subsequently, in July and thereafter, a parvovirus also was found that was antigenically related to feline panleukopenia virus (10). The pathology of the latter infection was remarkably similar to that of the feline disease. In August and September 1978 two United States government sponsored laboratory dog colonies had outbreaks of diarrheal disease and laboratory assistance was requested of the Division of Veterinary Medicine of the WRAIR. This summer, a further request for laboratory assistance was received from the WRAIR SEATO laboratory in Bangkok, Thailand. This report summarizes studies conducted on the etiology of these outbreaks. In addition, observations are summarized on the pathogenicity of a canine coronavirus obtained from WRAIR laboratory dogs with fatal diarrhea.

a) Virus studies of diarrheal disease among laboratory beagle pups at Raltech Scientific Services, St Louis, MO.

The US Army has a long term program to determine the wholesomeness of irradiated foods at the Raltech Scientific Services (RSS), Ralston Purina Company, St Louis, MO. The studies are monitored

by the Office of Wholesomeness of Irradiated Foods (OWIF) in the Headquarters of the US Army Medical Research and Development Command (USAMRDC). For these purposes, laboratory Beagle dogs are being used as an experimental animal. During June through August 1978, diarrheal disease with increased mortality occurred in pups at RSS and virus studies to determine the etiology were requested of the Division of Veterinary Medicine of the WRAIR by the USAMRDC. Virus isolation and blood specimens for serological tests were obtained from affected pups and bitches and processed as previously described (14).

Virus isolation tests were conducted on throat and rectal swab specimens of 18 pups from 4 litters with signs of diarrheal disease and 3 bitches which had or previously had diarrhea. Each specimen was treated with antibiotics, centrifuged and inoculated into dog kidney, canine A-72, and Walter Reed canine cell cultures. Transmissible agents were not recovered.

In addition, serological tests were done to detect viral infections on paired serum specimens of 12 Beagle bitches, 9 of which had a litter with diarrhea or enteric disease. Serum specimens from the pups with diarrhea were not available for examination. Individual serum specimens were examined also from the 13 other Beagle bitches with a history of pup mortality in their litters without diarrhea.

Serum neutralizing antibodies to the canine coronavirus (CCV), transmissible gastroenteritis virus (TGE) and canine herpesvirus were detected in nearly half the specimens tested (Table 10). Although, 6 of the dogs with diarrheal disease had coronavirus antibody, only 1 dog had a greater than 4-fold increase in titer to both CCV and TGE. Three other dogs had equivocal rises in titer to either or both of these viruses. Neither neutralizing antibody to canine parainfluenza virus nor hemagglutination-inhibition (HI) antibody to canine panleukopenia virus were found.

Although the findings summarized in this report do not implicate any virus as the principal cause of increased mortality associated with the diarrheal disease. The increased antibody titers to CCV and TGE found in 1 dog indicate that this dog was infected with an antigenically related coronavirus about the time the diarrheal disease occurred. The presence of coronavirus antibody in nearly half of these RSS dogs indicates that infections with this virus occurred frequently. However, further studies will be required to relate these infections with the diarrheal disease in the puppies and dogs.

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Canine panleukopenia and parainfluenza viruses antibodies were not detected in any of the serums tested. The dogs at RSS are susceptible to these viruses. Introduction of the former virus will result in an epizootic of diarrheal disease and the latter respiratory disease. It is recommended that isolation of the study dogs and review of staff practices be continued to prevent the indirect introduction of any of these agents.

b) Studies of diarrheal disease in dogs at Argonne National Laboratory

Argonne National Laboratory (ANL) is a part of the US Department of Energy and conducts long term studies on the effects of low-level protracted irradiation in Beagle dogs. The dogs are maintained for their life spans to determine late effects. Therefore, each death to extraneous causes represents a great loss in effort, money and data. During late August thru October 1978 an extensive epizootic of gastroenteric disease occurred at ANL (15). More than half of the 800 dogs had diarrhea and 10 died. The illnesses were of unknown etiology and posed a significant threat to their research program. Initial observations made by Dr. L.E. Carmichael indicated that a parvovirus possibly related to the minute virus of canines might be involved (15). Dr. Carmichael referred the workers at ANL to the Division of Veterinary Medicine, WRAIR because of their extensive experience with these fastidious viruses. Accordingly, a request for diagnostic virus assistance was made to this Division. Of further interest was the occurrence of several cases of diarrhea in the laboratory workers associated with these dogs. This report summarizes the laboratory studies on specimens from the ANL dogs and personnel.

From a pooled fecal specimen of ANL dogs with diarrhetic disease a transmissible agent was recovered in feline kidney and dog A-72 cell cultures. Intranuclear inclusions were evident in infected cells. The agent hemagglutinated swine and rhesus monkey erythrocytes at 4° C. The hemagglutination was inhibited by feline panleukopenia virus antiserum but not by antiserum against the minute virus of canines. During this period a number of other laboratories reported the recovery of similar feline panleukopenia-like viruses from dogs. (10,11,12,13)

Serologic studies were carried out on acute and convalescent serum specimens from more than 30 dogs. Most of the dogs were receiving "low" levels of cobalt irradiation. Included in the group were dogs which had signs of disease early and late in the epizootic, as well as a small number of dogs which did not exhibit signs of disease. In addition, paired blood specimens approximately 30 days apart were collected from laboratory personnel with and without diarrheal disease.

To study the extent of the virus infection in the ANL colony a hemagglutination-inhibition test (HI) was developed (17). The test employed rhesus erythrocytes a pH of 5.8 and an incubation temperature of 4° C. To remove nonspecific inhibitors the serums were treated with 10% kaolin and subsequently adsorbed with 50% rhesus erythrocytes to remove the blood cell agglutinins. The reduced amount of kaolin from the customary 25%, resulted in a smaller loss of specific antibody from our reference serums. Procedures for other virus antibody tests have been described (14).

Nearly 80% of the dogs had a rise in titer to the ANL canine panleukopenia-like virus (Table 11). The same dogs had similar rises in titer to the feline panleukopenia virus. Only 1 unaffected dog did not have antibody in the convalescent serum specimen. Six of the 7 dogs which did not have a rise in titer had high antibody titers in both the acute and convalescent serum specimens. Overall, 97% of the dogs examined, had panleukopenia antibody. In addition, nearly one-third of the irradiated dogs had a rise in titer to the canine coronavirus and almost half to canine herpesvirus. The rises in herpesvirus antibody occurred primarily in dogs without homologous antibody in their acute serum specimens, i.e. 9 of 13 seronegative dogs had increased titers in contrast to 1 of 9 seropositives. Serological tests for canine distemper, canine hepatitis, parainfluenza viruses and the minute virus of canines did not reveal any concurrent infections. The failure to detect increased antibody to the minute virus of canines further served to differentiate the ANL parvovirus-isolate from the minute virus of canines.

None of the 21 laboratory workers, including 4 with diarrheal disease, had or developed antibody to the canine or feline panleukopenia viruses or to the canine coronavirus. In tests, done at the NIAID, NIH, Bethesda by Dr. S. Greenberg and A. Kapikian, no increased antibody to the "O" rotovirus, Norwalk agent, human coronaviruses OC45 and 229E, mouse hepatitis or calf coronavirus were detected. Thus, the etiology of the

laboratory workers diarrheal disease was not related to the canine panleukopenia, coronavirus or to other human viruses associated with diarrhea.

The serologic findings were consistent with and confirmed the electron microscopic and isolation observations reported by Eugster (18) and Appel (10). In other outbreaks of canine diarrhea, multiple viral infections are not infrequent and the severity may range from inapparent to fatal. However, the pathology and high communicability of the canine panleukopenia virus indicate that this agent was of prime importance. Further studies are required to determine the persistence and health significance of the virus in the colony and impact on the experimental work.

c) Studies of Epizootic canine diarrhea in Bangkok, Thailand.

During the summer of 1979, an epizootic of diarrheal disease "ravaged" the dogs in Bangkok, Thailand. Initial electron microscopic observations in Bangkok revealed the presence of parvoviruses in 2 of 6 stool specimens. At the request of LTC R.E. Whitmire, VC and MAJ D.S. Burke, MC of the WRAIR SEATO Laboratory, collaborative studies have begun to determine the etiology of the diarrheal disease. This report summarizes the initial findings at the Division of Veterinary Medicine. The procedures employed were described above (section 5b).

Initial work was direct to detect viral hemagglutinins in stool suspensions of affected dogs. High titer, 1:5,120 to 1:20480, rhesus erythrocyte agglutinins were detected in stool preparations of 3 of 24 dogs. The hemagglutination was inhibited in each instance by specific goat anti-feline panleukopenia serum. In addition, one low titering hemagglutinating stool suspension from a fourth dog also was inhibited by the same antiserum. These findings indicated the presence of a virus antigenically related to feline panleukopenia virus in the stools of affected dogs. To confirm these observations and detect other agents, virus isolation studies are in progress.

Paired serum specimens from the Bangkok dogs were examined for canine panleukopenia virus (Argonne isolate) hemagglutination-inhibition (HI) antibody. Prior to testing, the serums were adsorbed with kaolin and rhesus monkey erythrocytes. More than 85%, 55 of 63 dogs had significant HI antibody in either or both serum specimens. Only 2 dogs had a rise in titer to this virus.

These observations are consistent with the rapid rise in antibody titers that occurs in this infection and time of collection of the first serum specimens. Additional serological tests are in progress to detect infections with the canine coronavirus.

The initial observation summarized in this report clearly indicate the presence of canine parvovirus in dogs from Bangkok. Both viral hemagglutinins and HI antibody were found in the stools and serums of dogs with diarrheal disease. The findings provide additional data on the significance of this virus in canine diarrheal disease and extend our knowledge on the distribution of the virus.

- d) Pathogenicity of a canine coronavirus recovered from laboratory dogs with fatal diarrhea.

The previous annual report summarized the recovery and identification of a coronavirus from laboratory Foxhound pups with fatal diarrheal disease. Due to the severity of this infection pathogenicity tests in dogs were undertaken to determine whether the isolate possessed unusual virulence. The present report summarizes the clinical and virus studies.

To determine the pathogenicity of the coronavirus 17B8 isolate, three 10-week-old foxhound dogs were given per os approximately 100,000 tissue culture infectious dose₅₀ of the virus. Mild enteric signs were evident on days 4 and 5 in 2 animals. They had loose stools with some mucous present (Table 2). The coronavirus was recovered for 5 to 10 days from rectal swab specimens of each dog. In addition, each dog developed serum neutralizing antibody to our reference 1-71 canine coronavirus. The findings made in this study are compatible with earlier studies of canine coronavirus infection in younger puppies and do not indicate any unusual virulence with this isolate.

Further studies are required to determine the factors which potentiate the pathogenicity of canine coronavirus infections.

6. Viral studies on WRAIR feline colony at Ft Meade, MD.

The WRAIR farm colony was established to provide investigators with high quality laboratory cats which could be employed in long term studies. Although the cats produced by the colony have met the needs of the investigators, there remains a shortage due in part to conjunctival and respiratory disease in nearly all the cats, and an overall kitten mortality rate greater than 50%. Many dead kittens are athymic, suggestive of immunosuppressive viral infection. This report summarizes initial virus laboratory studies to determine: 1) the etiology of disease, 2) serologic surveys to detect the immune status of the breeding stock to feline panleukopenia virus and, 3) detect antibodies to feline peritonitis virus. The findings are evaluated and future studies are discussed.

The colony population consists of approximately 90-100 mixed-breed, short-hair domestic cats (8-10 toms, 60-70 queens, and a variable number of young kittens and cats from litter production for colony replacement or issue). The colony is maintained in a non-barrier, open management system, housed in concrete block farm-type buildings with indoor-outdoor enclosed areas for six to eight different breeding groups, plus separate queening and post-weaning areas. Source animals are screened for feline leukemia virus, and then integrated into the colony after proper immunization for feline panleukopenia (FPL), feline rhinotracheitis (FVR), and feline calicivirus (FCV); as well as general physical examination and treatment for internal and external parasites.

Statistics compiled for the period of November 1978 through mid-July 1979 show that a total of 58 litters were produced from a group of 46 queens. 233 kittens were born, with the average litter size of 4 (range 1-8), and overall kitten mortality pre- and post-weaning being 59%. Mortality varied between a maximum of 96% in the coldest month (February litters), to a minimum of approximately 30% for June litters (the reporting period ended on 12 July, therefore, statistics are incomplete for June pre- and post-weaning mortality).

Major pathology diagnosis for mortality cases during the period included feline panleukopenia, feline infectious peritonitis, toxoplasmosis, pneumonia, pneumonitis, nephritis, enteritis, and conjunctivitis. Of particular importance is the frequent gross finding of athymic kittens indicating an underlying immunosuppressive factor such as is found with pre- or postnatal infection with feline infectious peritonitis (FIP), or feline

leukemia (FeLV) viruses (22).

a. Virus isolations on mortality cases - WRAIR Feline Colony.

Using standard isolation and identification methods, tissues from several necropsies yielded a herpesvirus similar to FVR, and also FCV, both viruses of which can play a major role in respiratory disease of cats and mortality in kittens. Pathogenic strains of both viruses have been shown to be intermittently shed (FRT), or persistently shed (FCV), by some adult cats. These cats often have a low to intermediate level of protective immunity to natural infection (20). Further studies are necessary to characterize this "immune carrier state" in the WRAIR colony, and assess the need for a modified vaccination program.

b. Survey of the WRAIR feline colony for serum antibodies to feline panleukopenia virus.

From 20 October through 15 December 1978 serum samples were collected from 44 selected breeding cats in the colony at Ft Meade, MD. Table 13 summarizes the results of a hemagglutination-inhibition (HAI) test modified for use in our laboratory. (21,23,24)

As shown in Table 13, cats were grouped according to the three main acquisition periods. This was done to compare the responses to the differing vaccination schedules. Cats in group A with the lower titers ($\geq 1:80$) had not received FPLV vaccine since August 1976. Those with higher titers (1:160-1:640) had received their last boosters in September 1976. Overall group A cats are the oldest group (age range 2-3 years), and exhibited the greatest range and distribution of immunity to FPL virus.

Group B cats, of which 88% had titers of less than 40, had either no record of vaccination with FPLV vaccine, or only one immunization. In reviewing the breeding records of cats with the lowest breeding to conception ratios prior to the survey, their titers to FPLV were significantly lower than queens who had conceived. This could point to the probability of an immune carrier state in queens (19) with lower titers, and/or a greater susceptibility to natural infection during pregnancy, resulting in early resorption or undetectable abortion of litters.

Group C cats, (most recently acquired group surveyed) were immunized with FPL virus upon entry into the colony, and correspondingly showed the highest titers as a group.

In summary, the results of the survey indicated that one-third of the queens had HAI titers to FPLV of 40 or less. Accordingly their kittens would have greatly diminished protective level of passive immunity (28). A systematic revaccination and booster program has been implemented. Re-evaluation of immune status and response to the immunization schedule is planned to continue during the next reporting period.

Table 14 shows a direct correlation between HAI and neutralization titers, confirming the validity of the HAI method as modified for use in our laboratory.

c. Pilot serologic survey for antibody to feline infections peritonitis virus.

The occurrence of mild, chronic or enzootic upper respiratory, conjunctivitis, and keratitis problems has approached 100% in the colony. In addition to FCV and FVR contributing to the problems, it is interesting to observe that the clinical syndrome caused by initial exposure of cats to the coronavirus of FIP, is essentially that of a transient upper respiratory and conjunctival infection. Since a pathologic diagnosis of FIP has been recorded in the colony, there is a high likelihood that FIP is also an enzootic problem. Isolation methods for this virus are complex, costly and in many cases unsuccessful. Serologic methods are under investigation to confirm the presence of this agent in the colony.

Selected sera from the previously described surveys were also tested by indirect immunofluorescence (IFA test) to test colony cats for antibody to the coronavirus of FIP. This agent has been shown to be present in many closed and open colonies/catteries throughout the world, and has been strongly implicated in epizootics of kitten mortality in colonies (29). A heterologous IFA test was adopted with TGE coronavirus antigen grown in WRAIR A-72 cells.

All test sera from colony cats gave positive fluorescence in a titer range from 1:6.25 to 1:100, which indicated low to moderate titers. (Table 15). While a very limited possibility exists that titers reflect colony infection with TGE virus, it is most probable

that the titers are due to exposure to FIP coronavirus, in which case a neutralization test against TGEV would be negative (26, 27). Such confirmatory tests are currently being prepared.

Summary.

Viral respiratory and enteric disease appear to be a major problem contributing to the morbidity and mortality in the WRAIR feline colony. The isolation or histopathologic evidence of FVR virus, FCV, and FPL virus from necropsy cases, coupled with the knowledge that each of these viruses can exist in the carrier state, points to the need for: 1) further isolation studies, 2) a more intensive immunization program, along with serosurveillance measure to insure its efficacy, 3) further investigation of animals which are possible "immune" carriers, 4) efforts to reduce all possible sources of stress or immunosuppressive factors. With regard to the latter point, periodic following studies are indicated to detect and eliminate possible carriers of FIP, FeLV and FPLV from the colony.

Summary:

Two years of epizootiology, infectious disease diagnosis and surveillance were compared in one year segments of October 1977 to September 1978 and October 1978 to September 1979. Over this two year period, K. pneumoniae associated deaths have decreased from 52% to 6%.

In the rat colony, K. pneumoniae associated abscessed rats have been culled from the colony, but there are still sporadic cases found. C. kutscheri has been isolated from dead stressed rats, but not unstressed surveillance tested rats. P. pneumatropica has recently been isolated from rats, guinea pigs and mice in our colonies and poses as similar a threat as C. kutscheri. Salmonellosis has been found in the guinea pig colony, but strenuous Testing and culling has reduced the culling rate to zero because no Salmonella sp. have been isolated in the last 3-4 months. Viral serologies of the rodent colonies indicate that the barrier systems are in need of repair.

New media and techniques have been devised to detect small numbers of K. pneumoniae.

Resistance transfer factors have been found in K. pneumoniae isolates cultured from Aotus monkeys.

Further virus studies have been conducted on the post mortem tissues of Aotus monkeys. Cytomegalo-like viruses were recovered from salivary glands and throat specimens from approximated half the monkeys examined. Adenoviruses were recovered from 1 adult and 1 infant monkey's rectal swab specimens. Significant antigenic differences were found among each of the cytomegalo like viruses and adenovirus isolates examined. Inapparent measles infections were detected in 3 WRAIR Aotus monkeys and approximately 20% of the colony had measles HI antibody. Hepatitis A virus antibody was also detected in the WRAIR colony and in 2 of 25 newly captured Panamanian Aotus monkeys. The significance of these observations are discussed.

Virus studies of diarrheal disease in laboratory dogs in the United States and dogs in Bangkok were carried out. Canine panleukopenia-like virus antibodies occurred in high frequency in laboratory dogs in the United States and Bangkok. Serological tests also indicated coronavirus infections were not uncommon in these spizootics. No serological evidence was found of canine

panleukopenia-like virus or coronavirus infections in exposed laboratory workers. The findings are evaluated and discussed. Further studies of the epizootics are in progress.

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Presentations:

1. Bunte, R.M., Beattie, R.J., Marchwicki, R.H., Sims, R.E. and Binn, L.N. Fatal Enteric Disease In Foxhounds with Coronavirus Infection. 59th Conference of Research Workers in Animal Disease, Chicago, IL Nov 27-28, 1978.
2. Bunte, R.M. and Binn, L.N. Coronavirus Infection in Laboratory Foxhounds. 30th Annual Session American Association for Laboratory Animal Science. Atlanta, GA Sep 16-21, 1979.

Table 1: Antibody prevalence rates in retired breeder mice to select viral antigens from 1 August 1977 to 31 August 1978

Location	Dates	Number Positive/Number Tested (%) ^a		
		PVM	Sendai	MHV
Room 3	Aug-Oct 77 ^b	1/29 (3)	0/21 (0)	9/15 (60)
	Jan 78 ^c	0/10 (0)	0/10 (0)	0/10 (0)
	Feb 78	0/10 (0)	0/10 (0)	0/10 (0)
	Mar 78	0/10 (0)	10/10 (100)	0/10 (0)
	May 78	0/10 (0)	10/10 (100)	4/10 (40)
	Jun 78	0/10 (0)	10/10 (100)	5/10 (50)
	Jul 78	2/19 (22)	9/9 (100)	1/10 (10)
	Aug 78	0/10 (0)	9/10 (90)	0/10 (0)
Room 6	Aug-Dec 77	29/49 (59)	3/36 (8)	4/40 (10)
	Jan-Mar 78	19/30 (63)	24/30 (80)	9/10 (90)
	Apr-Jun 78	6/10 (60)	10/10 (100)	9/10 (90)
Room 8	Aug-Dec 77	30/50 (60)	39/49 (80)	3/46 (7)
	Jan-Mar 78	17/30 (57)	28/30 (93)	2/27 (7)
	Apr-Jun 78	9/20 (45)	20/20 (100)	6/20 (30)
	Jul-Aug 78	15/20 (75)	20/20 (100)	3/20 (15)

^aSera were uniformly negative for antibodies to Reovirus 3, GD-M, Ectromelia virus, Mouse adenovirus, and LCM virus.

^bMouse colony was terminated in December 1977.

^cNew mouse colony was established in January 1978.

Table 2: Frequency of K. pneumoniae isolates' resistance to antibiotics and natural mutation to sensitivity

Strain	Origin	Reaction Indole	Antibiotics			
			P	E	C	S
122	Necropsy	+	216/0	216/0	216/0	216/0
8071T	Throat	+	162/0	162/0	161/1	161/1
120	Necropsy	+	208/8	216/0	205/11	216/0
27	Rectal	-	214/2	216/0	210/6	216/0

Table 3: Transfer of antibiotic resistance from K pneumoniae and Sh flexneri transconjugates to E coli 3951 N^rS^r

Donor	X	Recipient	Recipient Antibiotic Profile	Agglut. <u>E coli</u> sera 3951
K pneumoniae	27g	E coli 3951	P ^r C ^S E ^r T ^S S ^r N ^r	+
	120i		P ^r C ^r E ^r T ^S S ^r N ^r	+
	8071T		P ^r C ^r E ^r T ^r S ^r N ^r	+
	122		P ^r C ^S E ^r T ^S S ^r N ^r	+
	27g	Sh flexneri 24570	P ^r C ^r E ^S S ^r T ^r N ^S	-
Sh flexneri	27gp ^r	E coli 3951	P ^r C ^S E ^r T ^S S ^r N ^r	+
	27p ^r		P ^r C ^r E ^r T ^r S ^r N ^r	+
Controls:				
K pneumoniae	27g		P ^r C ^r E ^r S ^r T ^r N ^S	-
	120i		P ^r C ^r E ^r S ^r T ^r N ^S	-
	8071T		P ^r C ^r E ^r S ^r T ^r N ^S	-
	122		P ^r C ^r E ^r S ^r T ^S N ^S	-
Sh flexneri	24570		P ^S C ^S E ^S S ^S T ^S N ^S	-
E coli	3951		P ⁺ / ⁻ C ^S E ⁺ / ⁻ T ^S S ^r N ^r	+
Sh flexneri	27gp ^r		P ^r C ^r E ^S T ^r S ^r N ^r	-

Table 4. Virus Isolations from Aotus Monkeys During 1978-79.

Monkeys Examined	Specimen	Number with Cytomegalo-like virus	
		Total Examined	(%)
Adult dead	Throat	2/20	(20)
	Air sac	0/21	(00)
	Salivary glands	11/21	(52)
	Lungs	0/23	
	Liver	0/23	
	Adrenal	0/8	
	Kidney	0/23	
	Spleen	0/23	
	Rectal	0*/22	
Infant dead	Throat	0/4	
	Air Sac	0/3	
	Salivary glands	1/4	(25)
	Lungs	0/5	
	Liver	0/5	
	Adrenal	0/1	
	Kidney	0/4	
	Spleen	0/5	
	Rectal	0*/4	
Adult apparently healthy**	Throat	4/5	(80)
	WBC	0/4	
	Urine	0/5	

*Adenovirus recovered

**Aotus monkeys examined 2 years after original recovery of cytomegalovirus.

Table 5. Cross serum neutralization tests with rabbit antisera and selected Aotus Cytomegalo-like viruses.

Antiserum* (Rabbit no.)	Serum neutralization titer to:		
	7933T	8003T	43T
7933T (914)	<u>128</u>	0**	16
8003T (80)	0	<u>32</u>	0
43T (283)	0	0	<u>1024</u>

* Homologous preimmunization serum titer 1:8

** 0 = less than 1:16

Table 6. Results of cross serum neutralization tests with the WRAIR Aotus adenoviruses and their rabbit antisera

Antiserum*	Serum Neutralization titer to:			
	8138R	WR32R	WR122R	Ao119R
8138R	<u>128</u>	64	0**	256
WR32R	0	<u>4096</u>	16	128
WR122R	0	32	<u>512</u>	1024
Ao119R	16	2048	64	<u>2048</u>

* Preimmunization titer less than 1:16

** 0 titer equals less than 1:16

Table 7. Results of serum neutralization tests between WRAIR Aotus adenoviruses and LAIR Aotus and related adenovirus antiserums.

Antiserum (Source-Titer)*	Serum neutralization titer to:			
	8138R	WR32R	WR122R	Ao119R
Aotus Adenovirus I (LAIR-1280)	0**	0	0	0
Aotus Adenovirus II (LAIR-2560)	16	0	0	32
Aotus Adenovirus III (LAIR-1280)	16	128	0	0
Aotus Adenovirus 8-274*** (LAIR-1280)	0	64	1024	512
Aotus Adenovirus 8-364*** (LAIR 640)	0	0	0	0
Squirrel Monkey Adenovirus I (SVRC-1280)	0	0	0	0
Rhesus Monkey Adenovirus SV11 (SVRC-640)	0	32	0	0
Homologous (WRAIR)	<u>256</u>	<u>2048</u>	<u>1024</u>	<u>512</u>

* LAIR = Letterman Army Institute of Research (Dr. Shroyer)
SVRC = Simian Virus Reference Center (Dr. Lester)

** 0 titer = less than 1:16

*** Untyped isolates

Table 8. Summary of measles virus hemagglutination-inhibition antibody surveys of the WRAIR Aotus monkey colony.

Dates Bled	Source of Monkeys	No. seropositive No. tested (%)	No. Converted
Jun - Aug '78	WRAIR bred	0/17 (0)	
Oct '78	Purchased	5/27 (18.5)	
	WRAIR bred	0/3 (0)	
Apr '79	Purchased	6/29 (20.6)	1
May '79	Purchased	6/40 (15.0)	1
	WRAIR bred	1/11 (9.1)	1
<hr/>			
Totals:	Purchased	17/84 (20.3)	2
	WRAIR bred	1/31 (3.2)	1

Table 9. Summary of Hepatitis A Virus antibody surveys of Aotus monkeys.

Monkeys examined	No positive/No tested (%)*	
WRAIR Procured	15/26	(57.8)
WRAIR bred	1/26	(3.9)
Panamanian newly captured	2/25	(8.0)

* HAVAB test (Abbot Laboratories) conducted by MAJ S. Lemon, MC, Dept Virus Diseases, WRAIR

** Serum specimens from newly captured monkeys obtained from Dr. J.S. Harper III of the Gorgas Memorial Laboratory, Balboa Heights, Canal Zone.

Table 10. Viral antibody studies of Beagle bitches at Raltech Scientific Services, St Louis, MO.

Dog No.	Date onset diarrhea	Antibody titer to virus					
		Canine Coronavirus (Neut)	Transmissible gastroenteritis (Neut)	Canine herpesvirus (Neut)	Canine parainfluenza (Neut)	Panleukopenia (HI)	Canine
<u>Diarrheal disease study group*</u>							
A005	15 Aug 78	0/0**	0/0	4/4	0/0	0/0	0/0
A008	8 Aug 78	16/16	4/≤4	0/0	0/0	0/0	0/0
A014	None	0/0	0/0	0/0	0/0	0/0	0/0
B001	None	0/0	0/0	0/≤4	0/0	0/0	0/0
B002	15 Aug 78	4/4	4/4	4/0	0/0	0/0	0/0
B003	21 Jul 78	16/16	16/16	4/≤4	0/0	0/0	0/0
B011	None	0/≤4	0/4	0/0	0/0	0/0	0/0
C001	18 Jul 78	16/16	0/≤4	0/0	0/0	0/0	0/0
C011***	25 Jul 78	0/≤4	0/0	0/0	0/0	0/0	0/0
D003	7 Aug 78	0/0	0/0	16/8	0/0	0/0	0/0
E012	15 Aug 78	≤4/≤4	0/0	0/≤4	0/0	0/0	0/0
E013	18 Jul 78	4/64	4/16	0/0	0/0	0/0	0/0

*15 Aug 78 serum/6 Sep 78 serum.

**0 = less than 1:4 in neutralization, less than 1:40 in HI.

***Hemorrhagic enteritis in 1 pup.

****6 Sep 78 serum.

Table 10. (Cont'd) Viral antibody studies of Beagle bitches at Raltech Scientific Services, St Louis, MO.

Dog No.	Date onset diarrhea	Antibody titer to virus					Canine Panleukopenia (HI)
		Canine Coronavirus (Neut)	Transmissible gastroenteritis (Neut)	Canine herpesvirus (Neut)	Canine parainfluenza (Neut)	Canine	
<u>Non-diarrheal neonatal mortality group****</u>							
A016		16	16	0	0	0	Not done
C002		0	0	0	0	0	"
C004		0	0	4	0	0	"
C006		0	0	8	0	0	"
C007		0	4	0	0	0	"
D012		4	4	4	0	0	"
D013		4	4	4	4	0	"
D016		0	0	0	4	0	"
E004		4	4	8	0	0	"
E006		0	0	8	0	0	"
E015		0	0	0	0	0	"
E016		0	0	0	0	0	"
E017		0	0	0	0	0	"
No. sero-positive/total (%)		12/25 (48)	11/25 (44)	11/25 (44)	0/25 (0)	0/12 (0)	

*15 Aug 78 serum/6 Sep 78 serum.

**0 = less than 1:4 in neutralization, less than 1:40 in HI.

***Hemorrhagic enteritis in 1 pup.

****6 Sep 78 serum.

Table 11. Viral antibody studies of Argonne National Laboratory dogs with diarrheal disease.*

Experimental Status	Clinical Signs	Number of dogs with rise in titer/total examined**		
		Canine Panleukopenia-Like Virus	Canine Coronavirus	Canine Herpesvirus
Cobalt 60 Radiation	+	17/22	6/21	9/20
(0.4-10 R/Day)	0	3/3	0/3	0/3
<hr/>				
None	+	3/4	0/4	1/2
	0	2/3	0/3	0/3
<hr/>				
Totals:		25/32 (78%)*	6/31 (18%)	10/28 (36%)

* Increased antibody titers were not detected to CDV, ICH, Canine parainfluenza virus and the minute virus of canines.

** Acute serum specimens were obtained before the fifth day of disease; convalescent serums approximately one month later.

*** Similar rises in titers were observed to the feline panleukopenia virus.

Table 12. Experimental Infection of Foxhound pups with 17B8 coronavirus isolate.

Pup No.	Enteric Signs	Virus Recovered (Rectal Swab)	SN Antibody Titer (VS 1-71 Coronavirus)			
	(Days)	(Days)	Day 0	Day 14	Day 21	Day 35
100	4, 5	1-5, 10	2	8	32	128
101	4 5	2-10	2	8	32	32
102	None	3,4,6,7	2	2	32	8

Table 13. Results of feline panleukopenia virus serology survey on WRAIR farm feline colony breeding cats, Fort Meade, MD, October - December 1978^a.

Group ^b	No. cats with HA titers of ^c							No. cats tested per group/Total in group (%)	Geometric Mean Titer
	<40	40	80	160	320	≥ 640			
Group A (Jul-Sep/76)	6	4	1	4	5	3	23/26 (88%)		80
Group B (May-Nov 77)	7	0	0	1	0	0	8/17 (47%)		<40
Group C (May 78)	0	1	0	2	1	9	13/17 (76%)		400
TOTALS	13	5	1	7	6	12	44/60 (73%)		100

^aHemagglutination inhibition (HAI) with Rhesus erythrocytes.

^bBreeding cats are grouped by dates of acquisition. All cats sera from queens except for one tom in Group B.

^cReciprocal of dilution.

Table 14. Feline panleukopenia virus serum micro-neutralization titers on selected WRAIR farm cats, compared to HAI titers, 4-15 December 1978.

Cat number	1/HAI Titer	1/SN Titer
52 (NR)	20	32
1493 (733)	160	32
1693 (392)	>640	>512
1705 (1035)	>640	>512
1864	160	128
Ref. Anti-FPLV	>1280	>512

Table 15. Indirect Fluorescent Antibody Screening Test for feline infectious peritonitis virus antibodies in WRAIR farm cats^a.

Date	Cat No.	Group	Titer = 1/Dilution			
			6.25	25	100	400
20 Oct 78	1388	A	+	+	<u>+</u>	-
20 Oct 78	1388	A	+	+	<u>+</u>	-
"	1483	A	+	+	<u>+</u>	-
24 Oct 78	1480	A	+	+	<u>+</u>	-
4 Dec 78	1493	A	+	+	-	-
24 Oct 78	50	B	+	+	<u>+</u>	-
27 Oct 78	6	B	+	+	<u>+</u>	-
"	44	B	+	+	+	-
"	52	B	+	+	-	-
16 Nov 78	1693	C	+	+	<u>+</u>	-
"	1696	C	+	<u>+</u>	-	-
15 Dec 78	Kitten 184		+	-	-	-
"	Kitten 185		+	-	-	-
9 Feb 79	Kitten F-03		+	<u>+</u>	-	-
26 Jan 79	Kitten (Q 44)		+	-	-	-
Jul 79	Ref Pederson Negative		-	-	-	-
Jul 79	Ref Pederson 1:100 (IFA)		+	+	-	-
Jul 79	Ref Pederson 1:1600 (IFA)		+	+	+	+

^aEmploying transmissible gastroenteritis virus - infected canine A-72 cells as the antigen.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION*	2 DATE OF SUMMARY*	REPORT CONTROL SYMBOL	
				DA OB 6444	79 09 30	DD FORM (AR) 636	
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY*	6 WORK SECURITY*	7 REGRADING*	8A DISC'D INSTR*	8B SPECIFIC DATA CONTRACTOR ACCESS	9 LEVEL OF SUM
78 10 01	H-Termination	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10 NO /CODES*	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY	62770A	3M162770A802		00		012	
b. CONTRIBUTING							
c. CONTRIBUTING	CARDS 114F						
11 TITLE (precede with Security Classification Code)*							
(U) Diseases of Military Dogs							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS*							
010100 Microbiology 005900 Environmental Biology							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
68 07		79 10		DA		C. In-House	
17 CONTRACT/GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE NA				PREVIOUS		b. FUNDS (in thousands)	
b. NUMBER*				FISCAL YEAR		c. FUNDS (in thousands)	
c. TYPE				CURRENT		d. FUNDS (in thousands)	
d. KIND OF AWARD:				79		90	
19 RESPONSIBLE DOD ORGANIZATION				20 PERFORMING ORGANIZATION			
NAME* Walter Reed Army Institute of Research				NAME* Walter Reed Army Institute of Research			
ADDRESS* Washington, DC 20012				Div of CD&I			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME Russell, Philip K., COL				NAME* Stephenson, E. H., LTC			
TELEPHONE 202-576-3551				TELEPHONE 202-576-2146			
21 GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Lamb, S. R., III, CPT			
				NAME:			
22 KEYWORDS (precede each with Security Classification Code) (U) Military dog; (U) Ehrlichia canis; (U) Canine; (U) Cell hybridization; (U) Peritoneal macrophage							
23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified by number precede text of each with Security Classification Code.)							
<p>23. (U) Investigate diseases and/or conditions affecting or associated with the military dog to enhance diagnosis, treatment, and control.</p> <p>24. (U) Conventional epidemiologic, pathologic, and microbiologic methods were employed; special procedures were developed as needed.</p> <p>25 (U) 78 10 - 79 09 Investigations were finalized of the use of somatic cell hybrids for propagation of Ehrlichia canis. Magnetic separation of phagocytic cells following ingestion of carbonyl iron resulted in a significant enhancement of the phagocytis population with a concomitant increase in the percentage of cells susceptible to E. canis. Serial passage of the selected subpopulation of hybrid cells, however, resulted in a rapid diminution in the percentage of susceptible cells. Characterization was finalized of a canine cell line derived from a canine tumor. The cell line, which has a chromosome modal 2n chromosome number of 92 to 93, is the in vitro host cell of choice for canine coronaviruses, causative agents of viral diarrhea. The indirect hemagglutination test and the enzyme-linked immunosorbent assay were evaluated for possible use in diagnosis of ehrlichial infections, and to provide a diagnostic procedure adaptable to field laboratory operations. Extensive nonspecific reactions were observed with both test procedures and was attributed to the antigen preparation used. Investigations under this project were terminated because of the lack of an alternate in vitro propagation system in lieu of primary canine peritoneal macrophages or peripheral blood monocytes. Continued research would necessitate the expenditure of large amounts of man-hours and funds for minimal return in research data. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.</p>							

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DD FORM 1498

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Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 012 Diseases of the Military Dog

Investigators:

Principals: LTC Edward H. Stephenson, VC

Associates: Leonard N. Binn, PhD; CPT Samuel R. Lamb, III, VC; Ruth H. Marchwicki, BS; SP4 Brian L. Ermeling; SP4 Richard A. von der Porten

Description:

Investigators to define, study, diagnose, and control known and potential infectious diseases of military dogs. A major effort is directed toward developing an alternate in vitro method of cultivating Ehrlichia canis in lieu of the primary canine peripheral blood monocyte or peritoneal macrophage. Additional studies concern the epizootiology, diagnosis, treatment, and control of other disease agents or conditions affecting the military dog.

During the reporting period, research activities have included:

- (1) attempts at cultivation of E canis in cells other than primary canine peripheral blood monocytes or peritoneal macrophages,
- (2) characterization of a canine cell line for viral studies, and
- (3) developmental studies of a serologic test for E canis that would be adaptable to field conditions.

Progress:

1. Somatic cell hybrids of canine peritoneal macrophages and SV40-transformed human cells: derivation, characterization and infection with Ehrlichia canis.

Investigations were finalized of the use of somatic cell hybrids for propagation of E canis (1). Somatic cell hybrids were obtained by fusion of canine peritoneal macrophages and SV40-transformed human skin fibroblasts (LNSV). A cell line (WRH-2) was established from a single isolated hybrid clone. The WRH-2 cell line has been serially passaged 60 times and has a population doubling time of approximately 24 hours. Karyotypic analysis showed the modal number of chromosomes to be 80, with a selective segregation of canine chromosomes. Expression of incorporated canine DNA was substantiated by cellular enzyme activities and antigen expression. The susceptibility of 5 to 7% of WRH-2 cells to E canis infection was associated with phagocytic properties of these cells. Magnetic separation of phagocytic cells following ingestion of carbonyl iron resulted in a significant enhancement of the phagocytic population with a concomitant increase in the percentage of cells susceptible

to ehrlichial infection. Serial passage of the selected subpopulation of hybrid cells, however, resulted in a rapid diminution in the percentage of phagocytic cells.

2. Establishment of a canine cell line: derivation, characterization, and viral spectrum.

Investigations of a new canine cell line were completed (2). A cell line, designated A-72, for virus studies was established from a tumor surgically removed from a female, 8-year-old Golden Retriever dog. Following explant culture, the cells have been serially passaged 135 times. A-72 cells have maintained a fibroblastic appearance and, at the 123rd passage, had a population doubling time of approximately 27 hours. Karyotypic analysis of the high passage cells showed the modal 2n chromosome number to be 92 to 93. Using starch gel electrophoresis for enzyme characterization, the electrophoretic mobilities of enzymes extracted from A-72 cells were identical to those of canine peritoneal fibroblasts and primary canine kidney cells. The A-72 cells were susceptible to infection with infectious canine hepatitis virus, canine adenovirus type II, canine herpes virus, canine parainfluenza virus, and canine coronavirus, but was not susceptible to canine distemper virus or the minute virus of canines. These cells have been particularly useful for studies of the fastidious canine coronavirus, as the commonly used primary canine kidney cells exhibit varied susceptibility to these viruses.

3. Indirect hemagglutination test and enzyme-linked immunosorbent assay for detection of humoral antibodies to Ehrlichia canis.

Serodiagnosis of Ehrlichia canis infections in dogs is readily accomplished using the indirect fluorescent antibody (IFA) test (3). The IFA test is quite sensitive and specific, but is limited by the requirement for sophisticated equipment. Further, the antigen for the IFA test is comprised of ehrlichia-infected monocytes or macrophages fixed to microscopic slides, and these preparations can not be shipped readily to other laboratories that do not have the capabilities to prepare canine monocyte or macrophage cultures and propagate E. canis. A serotest procedure, therefore, was needed that was adaptable to field laboratory conditions and which used reagents that were economically prepared, with a long shelf life and minimal refrigeration requirements. Development of such a serotest procedure for E. canis would allow the test reagents to be prepared in a central laboratory with the ability to propagate the agents for antigen, then transship the reagents to field laboratories for on-site serologic testing.

Two serodiagnostic procedures exhibited the desired characteristics sought for ehrlichial antibody evaluation, namely the indirect hemagglutination (IHA) test and the enzyme-linked immunosorbent assay (ELISA). Both of these test methods had been developed previously for detection of other rickettsial infections (4,5). The purpose of this study was to evaluate the IHA test and the ELISA for use in the detection of humoral antibodies to E. canis.

Indirect hemagglutination test. Ehrlichia canis infected cultures of primary canine macrophages were prepared as previously described (6). Cell cultures were harvested for antigen preparation when 85% or more of the detached cells exhibited intracytoplasmic inclusions of E. canis. The cell contents from 25 flasks (25 cm²) were used for each batch of antigen. Infected cells and extracellular ehrlichiae were pelleted at 10,000 x g for 10 min at 4 C, washed twice in phosphate-buffered saline (PBS, pH 7.2), and resuspended in 20 volumes of PBS. The cell-ehrlichiae suspension was placed in an ice bath and treated thrice with ultrasound at a setting of 100 watts for 10 sec, with a 30 sec interval between each treatment. Following ultrasound treatment, the suspension was divided into 2 aliquots: one was used to prepare erythrocyte-sensitizing substance (ESS) by the method of Chang et al (7), and the second was used as a particulate antigen. Both antigens were diluted with PBS to a concentration of 100 ug of protein/ml, as determined by the Lowry method (8). A qualitative determination of antigen specificity was made by the direct fluorescent antibody test. Sheep erythrocytes were gluteraldehyde-treated and sensitized to each of the antigen preparations using the methods of Shirai et al (4). Control antigen preparations were prepared similarly from uninfected canine macrophage cultures. Test sera used were obtained from 8 dogs, one without ehrlichial antibodies (negative) and 7 with ehrlichial antibody titers of $\geq 1:512$ as determined by the IFA test. Three of the positive sera were from dogs experimentally infected with the same E. canis strain used for antigen preparation. The IHA test was performed by the methods of Shirai et al (4), using sera diluted 2-fold from 1:10 through 1:2560.

Serial dilutions of each antigen preparation were tested against serial dilutions of positive and negative control sera. Hemagglutination was not observed for any antigen-antiserum combination after incubation at room temperature for 1 hr. Consequently, the test procedure was reevaluated using the concentrated antigen preparations (100 ug of protein/ml) and introducing the following procedural modifications: (a) addition of 0.4% bovine serum albumin (BSA) to the diluent; (b) incubation temperature of 4, 20, or 37 C; and (c) variation of diluent pH using 5.8, 6.8, 7.2, or 7.8. Addition of BSA hastened settling of the sensitized erythrocytes (2-to 4-fold), alteration of the incubation temperature had

a direct effect on the rate of erythrocyte settling with the rate being faster as temperature increased, and variation of the diluent pH had no detected effect. Regardless of the procedure conditions employed, specific hemagglutination by positive antisera was not observed. Additional studies are requisite to elucidate the appropriate conditions for performance of the IHA test for ehrlichial antibodies. Areas that must be explored include better methods of antigen preparation, such as selective sonication and density-gradient separation, to obtain purified ehrlichiae, and the use of tannic acid-treated erythrocytes in lieu of glutaraldehyde-treated cells.

Enzyme-linked immunosorbent assay. Antigens used were the same as those prepared for the IHA test, except the protein concentration was adjusted to 150 ug/ml. Positive control serum used was from a dog experimentally infected with the same E canis strain used for antigen preparation. The ehrlichial IFA titer of the serum was 1:1280. The ELISA was performed according to reported methods (9), using 2-fold antigen dilutions against sera at undilute, 1:5, 1:10, and 1:40.

The color reaction of the ELISA was read visually and scored 4+, 3+, 2+, 1+, or -, according to the intensity of purple-brown color observed. A 1+ was interpreted as a positive reaction. Definitive color reactions were exhibited by both positive and negative canine sera vs ehrlichial antigen preparations, but the end-point of reaction for the negative control serum consistently was 1 to 2 dilutions less than those for the positive reference serum. Reactions were similar when uninfected cell antigens were used, except that the positive color reactions were 2 to 3 dilutions lower than the reactions with the ehrlichial antigens. The test results essentially were identical for the 2 antigen preparations, ether-base extracted and particulate. These results suggested that ehrlichial antibodies were being detected, but that the sensitivity of the test procedure was being masked by extensive nonspecific reactions. The nonspecific reactions apparently were attributable to the antigen preparations used. Although the ehrlichiae were propagated in serum-free medium for the final 24 to 48 hr before harvest and were washed twice prior to antigen preparation procedures, the antigens were not sufficiently pure to yield a specific seroreaction. Undoubtedly, the cellular components contained in the antigen preparations were of sufficient magnitude to impart the observed nonspecific reactions.

Neither the IHA test nor the ELISA yielded satisfactory serologic results, using the described methodology. The principal deficiency appeared to be production of a suitable, pure antigen for each of the tests. Adaptation of the IHA test and/or the ELISA to detection of ehrlichial antibodies must await development of an alter-

nate cell system in which to propagate E canis whereby massive numbers of organisms can be obtained, and development of separation and purification of ehrlichiae from infected cells.

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 012 Diseases of the Military Dog

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Publications:

1. Binn, L. N., R. H. Marchwicki, and E. H. Stephenson. 1978.
Establishment of a canine cell line: derivation, characterization, and viral spectrum. Proc. 59th Conf. Res. Workers Anml. Dis.
2. Stephenson, E. H. 1979. Canine coronavirus infection.
Abstr. 116th Ann. Amer. Vet. Med. Ass. Meet.

Presentations:

1. Binn, L. N., R. H. Marchwicki, and E. H. Stephenson. 1978.
Establishment of a canine cell line: derivation, characterization, and viral spectrum. 59th Conference of Research Workers in Animal Diseases, Chicago, IL. (Nov).
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116th Annual American Veterinary Medical Association Meeting, Seattle, WA. (Jul).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a		2. DATE OF SUMMARY ^a		REPORT CONTAINING SYMBOL DD FORM 1498	
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10. NO / CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
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23. (U) Develop physiological means of interrupting malaria transmission through an understanding of factors affecting parasite infectivity in vivo and in vitro. Determine most effective means of inhibiting malaria infections in vertebrate hosts by injection of liposomes containing glycolipids. Develop models for cyclical transmission of African trypanosomiasis to facilitate utilization of large numbers of parasites in studying immune mechanisms. Realization of objectives may lead to prevention or control of malaria and trypanosomiasis in military troops.									
24. (U) Assay liposome preparations, lacking or containing antimalarial drugs for effectiveness in inhibiting malaria infections. Isolate different stages of the malaria parasite on density gradients for subsequent study in culture systems. Correlate recently developed cyclical transmission models for tsetse flies with physiological factors that affect fly trypanosome interactions. Evaluate a self mating strain of Anopheles balabacensis for use in laboratory transmission of malaria.									
25. (U) 78 10 - 79 09 Liposome preparations containing neutral glycolipids with a terminal glucose or galactose, when injected intravenously, prevented the appearance of erythrocytic forms of malaria in hosts injected one day previously with sporozoites. Very high dilutions of the liposomes were therapeutically effective, retaining half the effectiveness of undiluted liposome preparations. However, liposomes containing membrane glycolipids were not effective against the erythrocytic forms nor did they have a prophylactic effect in the inhibition of malaria. The major factors influencing fly infection rates have been identified. Hence, sufficient salivary gland infections are being obtained to facilitate immunological evaluation. For technical report, see Walter Reed Army Institute of Research Annual Report, 1 Oct 78 - 30 Sep 79.									

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 013 Biological studies of insect infection and disease transmission

Investigators

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Description

The major objectives of this work unit have been (1) the establishment of a self-sustaining colony of tsetse flies, (2) the development of a rodent/fly/rodent transmission model for Trypanosoma rhodesiense, (3) the use of liposomes to treat experimental malaria {in collaboration with the Department of Membrane Biochemistry}, (4) the culture and biology of the mosquito pathogen Nosema algerae in insect cell lines and (5) a cytogenetic study of important anopheline vectors of malaria.

Progress

1. Colony Establishment of the Tsetse Fly Glossina morsitans

The basic parameters for successful establishment of Glossina morsitans have been determined using a New Zealand rabbit host. The female fly populations in April 1979 reached ~ 3,400, 2,400 of which were breeding females. The population at this level was producing approximately 1,000 puparia per week, with 9.8% of the females producing a puparium each day. Mortality was also low, averaging 0.8% per day. The colony was self-sufficient both for maintenance of a large breeding population and for production of flies for infection studies.

At this point a failure in the low-temperature override system of the incubator occurred, killing some 3,000 females. Since the older flies were virtually eliminated, an attempt was made to adapt the newly-emerging flies to the in-vitro feeding on pig blood favored by the Tsetse Research Laboratory, Langford, England. The flies were fed 4 days per week on pig blood and 2 days per week on rabbits. The puparial production rate decreased to about 7.0% of breeding females while daily mortality averaged 1.7%. Although these figures were significantly poorer than the figures for

rabbit-fed flies, the population climbed to $\approx 1,700$ females by August. At about this time, the blood heating unit overheated, killing some 900 females, thereby forcing continued importation of flies from England. These problems could have been averted by installation of an audible alarm system, which is only now nearing completion.

With the knowledge that rabbit-fed flies do better in all respects than flies fed in-vitro on pig blood, the in-vitro technique was terminated in favor of the rabbit host maintenance system. It is believed that the time-inefficiency of feeding on rabbits can be overcome by utilization of a larger breed of rabbit. The Department of Animal Resources is now investigating the possibility of obtaining the Flemish Giant breed as a solution to this requirement.

2. Development of a Rodent/Fly/Rodent Cyclical Transmission Model

This aspect of the work has progressed well due to continued supply of flies from England, during periods of low fly populations in our own laboratory. The greatest single difficulty encountered during the first year of successful cyclical transmission was the extremely low infection rates in all developmental stations in the fly, viz. midgut (MG), proventriculus (PV) and salivary glands (SG).

Since that time many of the factors that influence infection rates have been determined. It is now conclusive that males develop higher infection rates than females and that males fed <24 h post-eclosion attain higher infection rates than males 24-48 h post-eclosion (Tables 1 and 2). This age factor has not shown statistical significance in females nor has there been any difference between males and females 24-48 h post-eclosion (Tables 1, 2 and 3). Flies fed <6 h post-eclosion (Test 19) developed higher infection rates than flies 6-26 h or 24-30 h post-eclosion (Table 4). However, chilling 24-30 h old flies for 21 h at 18°C resulted in these flies attaining higher infection rates than 6-26 h flies. Combining data of flies <6 h old with 6-26 h old fly data and comparing these to data from 24-30 h old flies resulted in loss of statistical significance (Table 5). This suggested that in most experiments, it was the flies <6 h old that accounted for the majority of the infections. It was also of interest that only 13.5% of females <6 h old fed while 41.5% of similarly aged males fed.

Another factor of importance was the finding that 24-48 h old flies fed on serum-free blood (serum replaced with 1% glucose in

physiological saline) obtained greater than expected infection rates (Tables 2 and 6). Feeding flies on culture forms also tended to minimize the age effect in both males and females (Table 2), possibly in part due to the absence of serum in the infective meal. A post-infection temperature of 25°C also produced higher infection rates than a 20°C post-infection temperature when flies were fed a serum free bloodmeal (Tables 2 and 7). By far the highest infection rates were obtained when flies were fed on procyclic-form parasites from culture. Using this method, up to a 23% SG infection rate was attained by male flies <24 h old (Table 2). The method of infection evaluation was also found to be significant. Histological evaluation consistently resulted in lower infection rates than evaluations by dissection (Table 8). This limited the kinds of comparisons that could be made between tests 13-16 (histologically-evaluated) and 17-22 (evaluated by dissection).

Although the determination of factors that influenced infection rates were most important because infection rates could be maximized, a knowledge of factors that did not influence infection rates was also deemed important. Using bloodstream-form parasites to infect flies, post-infection temperatures between 15 and 21°C did not significantly alter infection rates. Also, feeding flies on a previously, cyclically-transmitted isolate (MFM) or on a clone (WRAtat 1) derived from the original LVH-18 isolate did not significantly alter the infection rates.

A number of other findings relating to development of the parasite in the fly were also discovered in the course of these experiments. For example, it was found that some flies which produced trypanosomes in their saliva did not have SG infections. Histological preparation of these flies showed that trypanosomes were, however, present in the esophagus and cibarial pump. Some flies also appeared to have immature SG infection that would not infect mice. These cases were thought to represent early post-invasion infection where trypanosomes had not yet developed into metacyclic forms. On the other hand, some flies with heavy SG infections did not infect irradiated mice as late as 4 days prior to dissection, a point when one would have expected at least some metacyclic forms to be present. Since cloning of metacyclics in mice has shown that a single metacyclic trypanosome is capable of infecting a mouse (and most probes produce hundreds or even a thousand or more trypanosomes), no explanation for the failure of some flies to infect mice could be ascertained. Occasionally, a fly which had previously infected a mouse was dissected and found clear of trypanosomes in the salivary glands. Either the SG infection was lost or trypanosomes from the PV may have infected the mice. However, injection of PV-form parasites into mice from 6 differently aged flies (ranging

from 14-36 days post infection) has not yet produced any parasitemic mice.

Harvesting parasites from salivary glands of many flies has shown an average harvest to be $\approx 10^5$ parasites, although many parasites remain attached to the gland epithelium. Observations to date have shown that all SG infected flies also have concurrent PV and MG infections. Flies with established MG infections (>10 days post infection) generally have heavier infection in the anterior MG than in the posterior MG. Of the hundreds of observations on MG-infected flies, none have shown infections in the hindgut (HG). It would appear that the unfavorable environment of the HG is sharply drawn at the MG-HG juncture and that such unfavorable condition often extend into the posterior MG.

Although many of the factors that influence infection rates are now known, the underlying bases for their operation are not understood. A complete understanding of these factors in the laboratory could permit a better correlation with the same factors as they operate in the field situation. This should facilitate determination of exactly which sets of conditions in the field contribute to increasing infection rates and eventually to epidemics.

3. The Use of Liposomes to Treat Sporozoite Induced Malaria (in collaboration with the Department of Membrane Biochemistry, Division of Biochemistry)

Liposomes are phospholipid bilayer vesicles that are taken up by the reticuloendothelial system following intravenous injection and are preferentially localized in the liver and spleen. In 1978, Alving et al., demonstrated that liposome-encapsulated antimonial drugs were far more effective and much less toxic than the drugs by themselves in treating experimental leishmaniasis in hamsters. Since the initial stages of sporozoite induced malarial infections are confined to the liver cells, the purpose of the present investigation was to test the effectiveness of liposome-encapsulated antimalarial drugs in suppressing malarial infections. Surprisingly, it was discovered that liposomes containing certain membrane glycolipids, without the inclusion of drugs, were capable of interfering with the life cycle of the malarial parasite.

Plasmodium berghei malaria was cycled through Anopheles stephensi mosquitoes and Golden Syrian hamsters. Sporozoites were harvested from the salivary glands of the mosquitoes 18-24 days after the latter had been given an infective bloodmeal. The experimental animals (ICR mice, Walter Reed strain) were each given 10,000 sporozoites intravenously 24 hours before injection of the liposome preparation. The liposomes consisted of dimyristoyl

phosphatidylcholine, cholesterol, dicetyl phosphate and a glycolipid in molar ratios of 1:0.75:0.11:0.12. Seventy nine percent of untreated control animals (saline injected) developed a patent infection, usually between day 4 and 6 after injection of the sporozoites compared with 7-15 percent of the animals injected with liposomes containing galactosyl, glucosyl or lactosyl ceremide. Liposomes lacking the carbohydrate group were ineffective as were ceremides having a strong negative charge attached to or near the terminal galactose. Encapsulating the antimalarial drug primaquine (which acts against the exoerythrocytic stages in the liver) into the liposomes may reduce the percentage of animals that become patent but, thus far, has not been successful in eliminating the infection altogether.

Liposome preparations effective against the liver stages were not effective against the erythrocytic stages as 100 percent of the mice injected with infective blood one day prior to injection with liposomes containing galactosyl ceremide became patent. Nor, in a single experiment, were the liposomes shown to have much of a prophylactic effect in inhibiting infection. Forty five of 58 mice (77 percent) injected with liposomes 1, 5 and 7 days prior to sporozoite injection became patent compared to 100 percent of the controls.

Finally, it is of interest to note that very high dilutions of the liposome preparations were therapeutically effective in that a 1:1,000,000 dilution of liposomes containing galactosyl ceremide retained half the effectiveness of undiluted liposomes. Additional details of this study may be found in a recent publication by Alving et al., (1979).

4. Culture and Biology of the Mosquito Pathogen Nosema algerae (Microsporida) in Insect Cell Lines

Microsporida are important disease agents of insect pests. One species, Nosema algerae Vavra and Undeen, has shown potential for the biological control of anopheline mosquitoes, particularly Anopheles stephensi and An. albimanus, in limited field trials. The microsporidium causes heavy mortality among larvae, reduced longevity and fecundity of surviving adult females and interferes with the development of Plasmodium within infected female mosquitoes. However, much basic information remains to be gathered about the biology and cytopathology of Microsporida in general and N. algerae in particular.

The current research, involving N. algerae in insect cell lines, concerns the acquisition or verification of data about the life cycle, cytopathic effects, intercellular transmission, host

specificity and mass rearing. Cell cultures, as opposed to intact insects, provide a simpler, better controlled host population in which the growth of the microsporidium may be easily observed. Furthermore, such in vitro systems may be manipulated to obtain physiological and biochemical information about the protozoan. Such a line of approach has been attempted only a few times with Microsporida.

In this study, N. algerae was reared in Heliothis zea (Corn ear worm) and H. virescens (tobacco budworm) following peroral infection of larvae. Doses of 3×10^5 spores per late first stage larva or 5×10^5 spores per second stage were optimal for host survival and spore production. Larvae were first starved for 24-36 hours and then offered spores in 10-20 μ l of water containing a minimal amount of larval diet as phagostimulant. With this procedure the gut was empty, reduced in diameter and thus presented less of a physical barrier to infection by the microsporidium. Spore production was further enhanced by rearing the infected insects at 20°C rather than 27°C. At this temperature insect development was greatly prolonged while N. algerae continued to multiply at near normal rates.

Previous techniques in which spores were harvested from surface sterilized pupae did not provide sufficient numbers of spores without a large number of insects and investment in time and labor. Infected adults, however, contained many more spores than pupae. Mass rearing of the microsporidium therefore was centered on harvesting spores from one-week-old adults each of which yielded approximately 10^9 spores. Spores reared in lepidopteran hosts do not lose their infectivity or virulence for mosquitoes.

Spore harvest and purification were accomplished by surface sterilization of infected adults followed by dissection of the fat bodies and musculature and homogenization in distilled water containing 1000 mcg gentamycin/ml. The resultant brei was cleared of larger debris by filtration through sterile cotton packed into a hypodermic syringe. Bacterial contamination was controlled by gentamycin during processing and storage. Fungal contamination was controlled by stimulating the conidia in the spore suspension to germinate by the addition of tissue culture medium. Twenty-four hours later the suspension was pelleted and resuspended in 200 mcg fungizone/ml. This process was repeated twice more. Finally the spores were purified in a discontinuous density gradient (40, 50, 60 and 70 percent DuPont Ludox in water), repeatedly washed by centrifugation and stored in distilled water with gentamycin and fungizone at 4°C.

Thus far, four insect cell lines have been employed, namely: MOS43 (An. stephensi), A.a. T43 (Aedes aegypti), TN368 (Tri-coplusia ni) and HZ1075 (H. zea). An. stephensi is the type host of N. algerae while A. aegypti is very refractive to peroral infections. Both lepidoptera will support growth of N. algerae especially when the spores are injected into the hemocoel.

Two million cells from log-phase cultures were suspended in 0.48 ml of Rinaldini's Salt Solution (RSS) containing an additional 1 mg KCl/ml, then mixed with 10^7 spores in 0.16 ml distilled water. Within 5 minutes 90-95 percent of the spores germinated. Aliquots of 0.1 ml were added to appropriate medium in plastic flasks or Leighton tubes and the cultures incubated at 25°C.

N. algerae established itself in all four cell lines. Infection rates were 50% for A.a. T43, 40% for MOS43, 50% for TN368 and 20% for HZ1075 after 24 hours (four replicates for A.a. T43, two for each of the other cell lines). About 60 percent of the HZ1075 cells lysed during and after the infection so that the 20% figure may not be representative of the H. zea line. The reason for the cell lysis is not clear but seems to be associated with penetration by spore polar filaments rather than the osmotic stresses during spore germination.

During preliminary work the earliest stages of N. algerae were not easily seen in host cells stained and examined with bright field illumination nor was phase contrast microscopy of living cells of value. The best observations have been with cell monolayers, washed in 80-90% strength RSS, air-dried, fixed with methanol and stained with Giemsa. An alternate technique is Heidenhain hematoxylin staining of Bouin-fixed cell monolayers. With such staining techniques N. algerae schizonts, 24-48 h post infection, appear as tiny diplokaryotic nuclei in the cytoplasm of their host cells. Cells grown on plastic coverslips appear to flatten more avidly to the substrate and hence are better material than those grown on glass for observing the microsporidium. Bi- and tetranucleate schizonts can be identified and counted best when the host cells are first exposed to a hypotonic solution before fixation and staining.

Infected cells in all four lines were rapidly outnumbered by uninfected cells. Seven days post-infection, the prevalence of infected cells decreased to less than 1%.

Further studies primarily concerned the A.a. T43 (A. aegypti) line. If these cells were grown in medium containing 1% serum as opposed to the normal 10% the growth rate of uninfected cells was

greatly slowed and the prevalence of infected cells decreased only slowly. Augmentation of the medium with serum and 2 mg l-glutamine/ml appeared to produce faster growth of the parasite in the host cells. Spore production began in A.a. T⁴3 cells on day 3 when glutamine was present versus day 7 when absent. This effect parallels the increased replication and assembly of nuclear polyhedrosis virus in lepidopteran cell lines when l-glutamine levels were elevated in the medium. The other cell lines have not yet been studied in detail.

Parasite numbers per A.a. T⁴3 cell reached 10-12 before sporoblasts appeared. In the smaller MOS43 cells parasite numbers reached 6-8 before sporogony. In both cases the host-cell cytoplasm was filled by the microsporidium and the cell was hypertrophied. Other workers have reported greater multiplication in the larger cells of Lepidoptera. These data may indicate that nutritional limitations, as reflected by host-cell size and crowding of the protozoans within the cell, initiate sporogony. Development of N. algerae was synchronous until sporogony at which time the infected host cells contained a variety of stages.

With continued cultivation, infections disappear from A.a. T⁴3 cell cultures after the third passage (two or three weeks after infection). Evidently there is little or no intercellular transmission of the microsporidium. This situation is quite different from the observations of other workers that the prevalence of infected Mamestra brassicae (noctuid moth) cells containing N. algerae remains the same over a number of passages, despite the rapid multiplication of uninfected cells. An extracellular stage has been hypothesized to explain the spread of microsporidian infections in insects. The only free stages of N. algerae observed in the A.a. T⁴3 cell cultures were those released by lysis of the host cell and these were infrequent. The released parasites remained clustered in the cell remains and showed no evidence of migration. In one experiment, examination of 600 infected A.a. T⁴3 cells 24-96 h post-infection did not disclose any evidence of microsporidia leaving a host cell.

5. A Cytogenetic Study of Important Malaria Vectors

Since the vector capacity of different anopheline mosquito species varies considerably, it becomes extremely crucial that reliable methods of identification become established before any efficient malaria control program can be initiated. Cytogenetics is one such potential method of identification. Variation in polytene chromosomal banding patterns are evaluated and then correlated to known morphological variants that have been studied by classical taxonomic techniques.

A. The ovarian nurse cell chromosomes of the neotropical subgenus Nyssorhynchus of Anopheles.

The subgenus Nyssorhynchus contains 22 species of which 5 (An. darlingi, An. allopha, An. albimanus, An. aquasalis and An. nuneztovari) are considered to be the primary vectors of malaria in Central and South America. The species in this subgenus are so closely related that their taxonomic picture has been confused for many years. Workers studying the malaria problem in Central and South America have never been sure just exactly what mosquito they were dealing with. To obtain a better taxonomic understanding of the subgenus, laboratory studies were initiated to determine the value of ovarian nurse cell chromosomes in the identification of the species in the subgenus.

Anopheles albimanus, the major vector in costal Central and northern South America and the Greater Antilles, was chosen as the first species to be examined because of the availability of existing colonies.

A colony originally from the Gorgas Laboratory in Panama was established and maintained as a source of adult female mosquitoes. A satisfactory means of preparing nurse cell chromosomes was developed. When adult females are 3-4 days old they are placed in small isolated cages and allowed to feed on anesthetized hamsters. The mosquitoes are then given a nutrient source and placed in an incubator at 15°C. At regular intervals (8, 16, 24, 32, 40, 48 and 56 h after blood feeding) the adults are preserved in Carnoy's 3:1 fixative. The ovaries are removed and each placed separately on a cover slip in a drop of 50% proprionic acid. The ovary is then dissected and the individual ovarioles separated. The ovarioles are left in the proprionic acid until they are swollen and gelatinous. At this time the proprionic acid is removed with filter paper and a drop of 0.5% lacto-aceto-orcein stain added. Excess stain is removed before adding a slide to prevent additional post-mounting staining. The slides are then ringed with nail polish and remain useful for several months. The preparations are routinely scanned with bright field illumination and well formed polytene chromosomes are easily visible at 100X magnification. Detailed examination and photography is best accomplished under oil and sometimes phase contrast illumination is helpful.

The most critical factor in the preparation of polytene chromosomes is the determination of the exact time after a bloodmeal when the nurse cell will be most active and the chromosomes well-developed. Mosquitoes, like most dipterans, have polytrophic, meroistic type ovaries and characteristically have 7 nurse cells surrounding a single oocyte. The nurse cells apparently contribute to yolk

deposition in the oocyte. The best preparations are obtained approximately 48 h (at 15°C) after a bloodmeal or when the ovaries are in Christopher's (1911) Stage III. The lowered incubation temperature appears to lengthen the critical period. If the ovaries are examined too soon after a bloodmeal (30-40 h) the chromosomes take on the appearance of giant lampbrush chromosomes similar to those seen in some panoistic-type oocyte nuclei (2). They almost appear to be polyploid in some cases. In fact, a characteristic feature of nurse cells of polytrophic ovarioles is their polyploidy (2). As time progresses after a bloodmeal the homologues pair, remain in close contact and replication continues until there may be 1000-2000 individual chromosomes in close apposition (3). At this time (48-50 h after a bloodmeal) the chromosomes are well formed and have a unique and distinct banding pattern as a result of variations in the tertiary structure of the DNA. It is at this time that the banding patterns can be mapped. If more time elapses after the bloodmeal the chromosome bands lose clarity and shortly thereafter (55 h after a bloodmeal) the chromosomes become amorphous.

An additional problem in finding the optimum time of examination is the tremendous variation observed in the time required for individual ovarian development. Normal development has been regarded to require 60-72 h at 25°C after a bloodmeal before eggs are laid. In the colony used in this study egg laying at 25°C required anywhere from less than 48 to as long as 120 h. When adult females kept at 25°C were dissected 24 h after a bloodmeal approximately 20% of the ovaries were not in Stage III. When females were kept at the lowered temperature of 15°C the ovaries developed at a more predictable rate and thus made finding the optimum time for dissection easier. At 15°C less than 5% of the ovaries examined at 48 h post-bloodmeal were not in Stage III.

The chromosomes are mapped by selecting preparations that are not excessively stretched by the mechanical stresses of the squashing operation and then starting to label the bands from the distal or free end of the chromosome. Because each arm consists of two homologous chromosomes tightly synapsed and twisted in a spiral along their length, the latter have a cross-section appearance of a figure-eight that leads to variations in the dilatations and constrictions (4). These features are also noted in addition to mapping the bands.

A map of the ovarian nurse cell chromosomes is now approximately 25% complete. The map should be useful even though the chromosomes of the nurse cells of An. albimanus are not as well developed as the nurse cells of many species of anophelines in the subgenus Cellia or even as well developed as the salivary gland chromosomes of An. albimanus.

The females examined in the subgenus Nyssorhynchus for nurse cell chromosomes will be identified by referring to recent revisions of the subgenus (5,6).

B. The larval salivary gland chromosomes of the neotropical subgenus Nyssorhynchus of Anopheles.

For the past 15 years or so, analysis of mosquito salivary gland chromosomes has been recognized as a valuable tool in the identification of closely related species. Some recent studies of the subgenus Nyssorhynchus have included the mapping of salivary gland chromosomes and the analysis of polymorphic populations. In order to further these studies a project was undertaken to examine a number of different colonies of An. albimanus from various geographical localities and try to analyze chromosomal variation in these populations.

The mosquitoes for this study consisted of 6 geographical strains. The first strain was part of the original Gorgas laboratory strain and was obtained from Dr. G.P. Georgiou at the University of California, Riverside. The other Panama strain was isolated in 1973 at Escobol in the Republic of Panama and was obtained from Drs. McWilson Warren and William E. Collins at the Center for Disease Control in Atlanta, Georgia. They also provided strains from Haiti, San Diego Norte in El Salvador, and Papayal, Colombia. The final strain from El Salvador was obtained from Dr. Jack Seawright at the Insects Affecting Man and Animals Research Laboratory in Gainesville, Florida.

The salivary glands were dissected from early fourth stage larvae as described by Jensen (7). The chromosome slides were prepared essentially by methods described by French, et al., (10). Preliminary chromosome maps were prepared after observation under oil immersion at 1000X and checked with the salivary gland chromosome map of Keppler et al., (9).

Preliminary comparisons indicate that there are no conspicuous banding differences between any of the 6 strains examined. More than 1500 slides have been examined and none have been heterozygous for any inversion. These results are in agreement with the findings of the previous studies of salivary gland chromosomes in albimanus (9). They are, however, in contrast to the findings in other species of Anopheles and especially those of the subgenus Nyssorhynchus (10). All species examined in the subgenus, except for albimanus, exhibit extensive variation in their chromosome complement with regard to the presence of paracentric inversions in various populations.

Single-pair mating experiments were conducted between each of the 6 strains. Results indicate that there is essentially complete interbreeding between strains. Furthermore, no inversion heterozygotes were observed in the F_1 generation in any of the crosses.

The fact that albimanus does not appear to establish paracentric inversions in its populations is not too surprising. The establishment of paracentric inversions may require that a special type of oogenesis, not yet observed in mosquitoes, occurs.

C. Mitotic and meiotic metaphase chromosomes of Anopheles albimanus.

The karyotype of Anopheles albimanus was first described from larval brains, ovaries and testes as having 2 pairs of metacentric and a pair of subtelocentric chromosomes (9). Recent examinations confirm this description.

The chromosomes were prepared following the methodology of French, et al., (8). All material was pretreated with colchicine to block cell division. Mitotic figures were obtained from late fourth stage larvae; meiotic figures from the testis of male pupae and ovaries of female pupae.

D. The ovarian nurse cell chromosomes of the leucosphyrus group of subgenus Cellia, genus Anopheles.

The increase in malaria transmission in the Oriental region has focused attention on the paucity of information regarding the vectors in the leucosphyrus group. Recently a new species (An. airus), has been described in the group that is probably the major vector of malaria in Thailand (11). In the hope to help elucidate the taxonomic problems of the group a cytogenetic examination of the Perlis strain of Anopheles balabacensis from Malaysia was begun. The strain was obtained from Ronald Rosenberg of NIH, Bethesda, Maryland.

The purpose of this study was to try to formulate a map of the adult female ovarian nurse cell chromosomes. The methodology for examining the Perlis strain was the same as that previously described for Anopheles albimanus.

Previous cytogenetic investigations using other species in the subgenus Cellia have indicated that the ovarian nurse cells contain large and well developed polytene chromosomes. This has not been confirmed in the present study for Anopheles balabacensis (Perlis strain).

The polytene chromosomes from ovarian nurse cells in An. balabacensis consist of 5 elements with a characteristic banding pattern. The X chromosome appears to be telocentric and has only one arm. The right and left arms of chromosomes 2 and 3 are subequal and moderately long. The banding patterns superficially resemble and previously described 17 species of subgenus Cellia. A detailed comparison will be attempted once the map of balabacensis has been completed. Additional strains of balabacensis will be examined and the ovarian nurse cell chromosomes mapped.

TABLE 1 Proportions of flies infected in midgut (MG), proventriculus (PV), or salivary glands (SG) as varied by isolates (LVH-18, cyclically-transmitted isolate = MPM, cloned isolate = WRatet 1), different pre- and post-infection temperatures, and varied fly ages (<24 or >24-48 h post-eclosion). Tests 13-16 evaluated histologically, Test 17 analyzed both histologically and by dissection, (See Table 2).

Test # (Isolate)	Parasitemia	Pre. Inf.	Post Inf.	MG				PV				SG			
				$\frac{\sigma^2}{<24}$	$\frac{\sigma^2}{>24}$	$\frac{\sigma^2}{<24}$	$\frac{\sigma^2}{>24}$	$\frac{\sigma^2}{<24}$	$\frac{\sigma^2}{>24}$	$\frac{\sigma^2}{<24}$	$\frac{\sigma^2}{>24}$	$\frac{\sigma^2}{<24}$	$\frac{\sigma^2}{>24}$	$\frac{\sigma^2}{<24}$	$\frac{\sigma^2}{>24}$
13 (LVH-18)	6.9 X 10 ⁷	28C	21C	5/42	4/12	7/41	0/4	2/41	2/12	5/50	0/4	2/41	1/10	0/50	0/4
			Prop. Inf.	.119	.333	.137	0	.049	.167	.100	0	.049	.100	0	0
15 (WRatet-1)	8 X 10 ⁶	28C	18C	5/34	3/32	4/43	1/24	3/31	1/32	2/42	1/24	3/28	0/27	0/42	0/19
			Prop. Inf.	.147	.094	.093	.042	.097	.031	.048	.042	.107	0	0	0
16 (LVH-18)	4.2 X 10 ⁷	28C	18C	9/49	3/59	1/17	2/34	7/48	3/56	1/17	0/27	1/48	1/55	0/16	0/27
			Prop. Inf.	.184	.051	.059	.059	.146	.054	.059	0	.021	.018	0	0
17 (MPM)	1.9 X 10 ⁷	28C	15C	4/27	1/9	2/20	2/18	3/19	0/9	1/20	0/17	0/11	0/8	0/14	0/13
	Histo.		Prop. Inf.	.148	.111	.100	.111	.158	0	.050	0	0	0	0	0

TABLE 2. Proportions of flies infected in midgut (MG), proventriculus (PV), or salivary glands (SG) as varied by isolates (MFM = cyclically-transmitted isolate, WRAtat 1 = cloned isolate), different pre-and post-infection temperatures, different forms of parasites (blood-stream or procyclic culture forms), varied fly ages (<24 or 24-48 h post-eclosion), and blood with or without serum. Test 22 consisted of females 24-32 days post-eclosion. All infections evaluated by dissection.

Test # (Isolate)	Parasitemia	Pre. Inf.	Post Inf.	MG			PV			SG					
				σ^7		ϕ	σ^7		ϕ	σ^7		ϕ			
				<24	>24	<24	>24	<24	>24	<24	>24	<24	>24		
17 (MFM)	1.9 X 10 ⁷	28C	21C	12/33	1/22	7/37	3/0	7/33	0/10	3/33	2/9	2/24	0/10	0/33	0/9
	Dissection	Prop. Inf.		.364	.083	.189	.333	.212	0	.091	.222	.083	0	0	0
18 (WRAtat 1 Procyclic)	3.1 X 10 ⁸	28C	15C	13/13	-	15/16	-	11/13	-	9/16	-	3/13	-	0/16	-
		Prop. Inf.		1.000	-	.938	-	.846	-	.563	-	.231	-	0	-
19 (MFM)	8.5 X 10 ⁷	28C	18C	31/101	10/47	10/42	13/77	29/101	8/147	7/42	11/77	8/91	1/40	1/31	3/72
		Prop. Inf.		.307	.213	.238	.169	.287	.170	.167	.143	.088	.025	.032	.042
20 (WRAtat 1 Serum-free)	2.1 X 10 ⁷	28C	20C	52/166	46/151	-	-	15/136	29/121	-	-	1/98	9/91	-	-
		Prop. Inf.		.313	.305	-	-	.110	.240	-	-	.010	.099	-	-
20C (Same)	Same	28C	25C	25/46	7/15	-	-	19/43	-	-	-	3/35	-	-	-
		Prop. Inf.		.543	.467	-	-	.442	-	-	-	.086	-	-	-
21 (WRAtat 1 Procyclic)	2.1 X 10 ⁸	28C	20C	43/61	29/36	34/42	14/17	38/55	13/29	26/34	5/7	10/49	4/25	1/20	0/7
		Prop. Inf.		.705	.806	.810	.824	.691	.448	.765	.714	.204	.160	.034	0
22 (MFM)	2.7 X 10 ⁷	27C	20C	-	-	-	3/54	-	-	-	0/44	-	-	-	-
		Prop., Inf.		-	-	-	.056	-	-	-	0	-	-	-	-

TABLE 3. Influence of age (< or >24 h post-eclosion) and sex of flies on midgut (MG), proventricular (PV), and salivary gland infection rates using data from pooled experiments (13-19, see Table 1). χ^2 significance levels as follows: 1+=.10, 2+=.05, 3+=.025, 4+=.010, 5+=.005

Factor Analyzed (Text #'s)	MG		PV		SG	
	$\bar{\sigma} < 24$	$\bar{\sigma} > 24$	$\bar{\sigma} < 24$	$\bar{\sigma} > 24$	$\bar{\sigma} < 24$	$\bar{\sigma} > 24$
Age (13-17,19)	Prop. Inf. χ^2 Signif. .201 3+	.130 0	.147 3+	.038 0	.040 0	.020 0
Sex (13-19)	$\bar{\sigma} < 24$ Prop. Inf. χ^2 Signif. .216 0	$\bar{\sigma} > 24$.177 0	$\bar{\sigma} < 24$.168 5+	$\bar{\sigma} > 24$.101 0	$\bar{\sigma} < 24$.054 5+	$\bar{\sigma} > 24$.012 0
Age between sexes (13-19)	$\bar{\sigma} < 24$ Prop. Inf. χ^2 Signif. .231 2+	$\bar{\sigma} > 24$.160 0	$\bar{\sigma} < 24$.187 5+	$\bar{\sigma} > 24$.093 0	$\bar{\sigma} < 24$.066 5+	$\bar{\sigma} > 24$.005 0
Age between sexes (13-17,19)	$\bar{\sigma} < 24$ Prop. Inf. χ^2 Signif. .134 0	$\bar{\sigma} > 24$.127 0	$\bar{\sigma} < 24$.084 0	$\bar{\sigma} > 24$.072 0	$\bar{\sigma} < 24$.020 0	$\bar{\sigma} > 24$.021 0
Age within a sex (13-17)	$\bar{\sigma} < 24$ Prop. Inf. χ^2 Signif. .192 2+	$\bar{\sigma} > 24$.106 0	$\bar{\sigma} < 24$.128 2+	$\bar{\sigma} > 24$.050 0	$\bar{\sigma} < 24$.052 0	$\bar{\sigma} > 24$.018 0
Age within a sex (13-17)	$\bar{\sigma} < 24$ Prop. Inf. χ^2 Signif. .140 0	$\bar{\sigma} > 24$.090 0	$\bar{\sigma} < 24$.093 0	$\bar{\sigma} > 24$.072 0	$\bar{\sigma} < 24$ 0	$\bar{\sigma} > 24$ 0

TABLE 4. Influence of age of flies (<6 h, 6-26 h, or 24-30 h post-eclosion) on MG, PV, and SG infection rates. Flies 24-30 h old were exposed to 1500 temperature for 21 h to curtail physiological aging. Data analyzed by analysis of means (Ott, 1967) to determine high (+), low (-) or normal (0) infection rates. UDL = 95% upper decision limit, LDL = 95% lower decision limit.

Infection Location	Sex Age	#Inf./Total	Prop. Inf.	UDL	LDL	Significance of Rate
MG	♂ <6	20/36	.556	.353	.201	+
	6-26	11/65	.169			-
	24-30	10/47	.213			0
	♀ <6	5/15	.333	.263	.123	+
	6-26	5/27	.185			0
	24-30	13/77	.169			0
PV	♂ <6	19/36	.528	.319	.181	+
	6-26	10/65	.154			-
	24-30	8/47	.170			-
	♀ <6	4/15	.267	.215	.087	+
	6-26	3/27	.111			0
	24-30	11/77	.143			0
BG	♂ <6	7/36	.194	.105	.025	+
	6-26	1/60	.017			-
	24-30	1/40	.025			0
	♀ <6	1/9	.111	.076	.002	+
	6-26	0/22	.000			-
	24-30	3/72	.042			0

TABLE 5. Influence of age of flies (<26 vs. 24-30 h post-eclosion) on MG, PV, and SG infection rates. Flies 24-30 h old were exposed to 18°C temperature for 21 h to curtail physiological aging. Data analyzed by χ^2 difference to show normal (0) or higher than normal (+, .10 χ^2 level) infection rates.

Location	Sex/Age	#Inf./total	Prop. Inf.	χ^2 Signif.
MG	♂ <26	31/101	.307	0
	♂ 24-30	10/47	.212	0
	♀ <26	10/42	.238	0
	♀ 24-30	13/77	.169	0
PV	♂ <26	29/101	.287	0
	♂ 24-30	8/47	.170	0
	♀ <26	7/42	.167	0
	♀ 24-30	11/77	.143	0
SG	♂ <26	8/91	.088	0
	♂ 24-30	1/40	.025	0
	♀ <26	1/31	.032	0
	♀ 24-30	3/72	.042	0

TABLE 6. Influence of serum-free blood (with 1% glucose/saline replacement) on MG, PV, and SG infection rates in ♂ flies <24 or 24-48 h post-eclosion. Data analyzed by χ^2 difference to show normal (0) or greater than normal (+ = χ^2 at .01 level)

Location	Age	#Inf./total	Prop. Inf.	χ^2 Significance
MG	<24	52/166	.313	0
	>24	46/151	.305	0
PV	<24	15/136	.110	0
	>24	29/121	.240	+
SG	<24	1/98	.010	0
	>24	9/91	.099	+

TABLE 7. Influence of post-infection temperature (20 or 20°C) on MG, PV, and SG infection rates in flies infected using serum-free blood with 1% glucose in physiological saline. Data analyzed using 2 differences to show normal (0) or greater than normal infection rates (+ = χ^2 at .025 level, 2+ = χ^2 at .005/level).

Location	Post. Inf. Temp. (°C)	Sex/Age	#Inf./total	Prop. Inf.	χ^2 Signif.
MG	20	♂ <24	52/166	.313	0
	25	♂ <24	25/46	.543	+
	20	♀ >24	46/151	.305	0
	25	♀ >24	7/15	.467	0
PV	20	♂ <24	15/136	.110	0
	25	♂ <24	19/43	.442	2+
SG	20	♂ <24	1/98	.010	0
	25	♂ <24	3/35	.086	+

TABLE 8. Influence of evaluation method (histological or dissection) on MG, PV and SG infection rates obtained for ♂ and ♀ flies <24 or >24 h post-eclosion (combined) tests 13-17 (histological) pooled and compared to tests 17 (dissection) and 19. Data analyzed by χ^2 difference with 0 = normal rate and + = χ^2 significance at .005 level

Location	Eval. Method	# Inf/total	Prop. Inf.	χ^2 Signif.
MG	Histo.	52/475	.109	0
	Dissection	87/358	.243	+
PV	Histo.	31/449	.069	0
	Dissection	67/352	.190	+
SG	Histo.	8/414	.019	0
	Dissection	15/310	.048	+

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 013 Biological studies of insect infection and disease transmission

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Publications

Alving, C. R., I. Schneider, G. J. Swartz, Jr., and E. A. Steck. Sporozoite-induced malaria: Therapeutic effects of glycolipids in liposomes. Science 205: 1142-1144, 1979.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)536	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY ACTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DISSEM INSTR ^a	9. SPECIFIC DATA - CONTRACTOR ACCESS	10. LEVEL OF SUM A. WORK UNIT
78 10 01	C. Change	U	U -	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
11. NO./CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62770A	3M162770A802	00	014			
B. CONTRIBUTING							
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11. TITLE (Precede with Security Classification Code) ^a							
(U) Characteristics of Attenuated Dengue Viruses							
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13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
75 07		CONT		DA		C. In-House	
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NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Precede with N.A.S. Academic Protection)			
NAME: Russell, Philip K., COL, MC				NAME: Harrison, Venton R.			
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				SOCIAL SECURITY ACCOUNT NUMBER: 73			
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Foreign intelligence not considered				NAME: Eckels, Kenneth H., Dr., Ph.D.			
				NAME: Summers, Peter L.			
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(U) Human volunteer; (U) Dengue; (U) Vaccine; (U) Immunity; (U) Tissue Culture							
12. TECHNICAL OBJECTIVE, 23. APPROACH, 24. PROGRESS (Precede individual paragraphs identified by number. Precede text of each with Security Classification Code)							
23. (U) The objective is development, production, and assay of live-attenuated vaccines against classical strains of dengue viruses. The major types (1,2,3, and 4) of this virus are endemic throughout populated areas of the world, and although mortality rates are low, the incapacitation effected by these viruses and their associated sequelae could have serious impact on military time tables and troop mobility.							
24. (U) Selected strains are subjected to multiple passages and frequent cloning in tissue culture systems, to produce pure progeny characterized by reduced virulence and adequate antigenicity, that will serve as candidate vaccine seed virus.							
25. (U) 78 10 - 79 09 1. Lot 1 of dengue-2 vaccine continued to be tested in groups of human volunteers. Non-flavivirus-immune individuals received doses of vaccine ranging from 100,000 to 100 PFU. A dose response pattern was not found and 8 of 19 vaccinees demonstrated seroconversion by standard tests. Five of these eight were viremic prior to antibody appearance. All virus isolates contained small plaque virus with no evidence of revertants. The frequency of dengue-like clinical symptoms was similar to that observed in the original study. 2. A radioimmunoassay (RIA) was developed to study the humoral immunoglobulin response to dengue-2 vaccine. IgG, IgM, and IgA immunoglobulins were detected in selected vaccinees. Antibody titers by RIA were compared with those titers found by standard tests. 3. A production seed for the C-5 vaccine candidate clone of dengue-3 was found to contain mixed viral populations showing varying degrees of temperature sensitivity. Failure to completely purify this clone or unstable genetic characteristics prevented further use of this C-5 line. Another line of the C-5 clone as well as other candidate clones are being characterized for future use. For technical report see WRAIR Annual Progress Report, 1 Oct 78 - 30 Sep 79.							

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 014 Characteristics of attenuated dengue viruses

Investigators.

Principal: Venton R. Harrison, M.S.

Associates: Kenneth H. Eckels, Ph.D.; Peter L. Summers;
Sp/5 Guy L. Tyndal

I. Dengue-2 Vaccine Progress.

A. Adsorption of vaccine virus. Growth of S-1 vaccine virus is invariably slower in terms of virus titer post inoculation than growth of the parent virus in cells inoculated with equivalent amounts of virus. A defect in adsorption may cause decreased replication and at higher temperatures of growth may account for some of the "ts" characteristics of the S-1 virus. Adsorption was looked at by infectious center assays. Cell suspensions or monolayers were inoculated with S-1 or parent viruses under conditions of varied temperature and pH. Adsorption in the presence of varying concentrations of NaCl were also done. Following adsorption, cells were washed and treated with DEN-2 antibody to neutralize extracellular virus. The cells were then diluted in diluent and plated on LLC-MK₂ monolayers and overlaid following plaque assay procedures.

Table 1 lists the effect of pH on adsorption of S-1 vaccine (lot 1) and parent (GM-6, FRhL-3) viruses to LLC-MK₂ cells. Both viruses adsorb better at the higher pH's of 7.8 and 8.1 than at pH 6.9 and 7.4.

Adsorption in the presence of increased (greater than physiological) concentrations of NaCl was done using similar techniques. Table 2 shows the effects of NaCl concentration to 1.5 M, ten times the physiological concentration. Both S-1 and parent viruses adsorbed to cells only in the presence of 0.15 M NaCl.

Temperature effects on adsorption were also examined for the two viruses. Suspensions of FRhL and LLC-MK₂ cells were made and conditioned in 23C, 31C, 35C, and 39.3C waterbaths. Viruses were inoculated and infectious centers calculated as above. The results (Table 3) indicate that the cell used for inoculation with S-1 virus is important for adsorption. Very little increase in infectious centers is seen for S-1 inoculated in FRhL cells at increasing temperatures, while a marked increase is seen in LLC-MK₂ cells. Parent virus adsorption is directly proportional to increased temperature in both cell types. The adsorption differences between S-1 and parent viruses are evident only in FRhL cells.

Table 1. Effect of pH on adsorption of S-1 and parent viruses to LLC-MK₂ cells.

Virus	pH	infectious ctrs/10 ⁵ cells
S-1 ^a	6.9	6.8 X 10 ¹
	7.4	6.6 X 10 ¹
	7.8	2.7 X 10 ²
	8.1	1.0 X 10 ²
parent ^b	6.9	2.8 X 10 ²
	7.4	3.1 X 10 ²
	7.8	3.7 X 10 ²
	8.1	4.1 X 10 ²

a S-1 inoc: 2.5 X 10⁴ PFU/0.2 ml
b GM-6 inoc: 2.2 X 10⁴ PFU/0.2 ml

Table 2. Effect of NaCl concentration on adsorption of S-1 and parent viruses to LLC-MK₂ cells.

Virus	M NaCl	infectious ctrs/0.2 ml
S-1 ^a	0.15	7.5 X 10 ¹
	0.5	0
	1.0	0
	1.5	0
parent ^b	0.15	2.2 X 10 ²
	0.5	1
	1.0	0
	1.5	0

a S-1 inoc: 1.8 X 10⁴ PFU/0.2 ml
b parent: 1.3 X 10⁴ PFU/0.2 ml

Table 3. Effect of temperature on adsorption of S-1 and parent viruses.

Virus	Cell	Temp	infectious ctrs/10 ⁴ cells
S-1 ^a	FRhL	23C	3.8
		31C	3.8
		35C	4.8
		39.3C	5.0
	LLC-MK ₂	23C	2.2
		31C	3.6
		35C	17.4
		39.3C	19.4
parent ^b	FRhL	23C	6.8
		31C	8.0
		35C	15.6
		39.3C	22.0
	LLC-MK ₂	23C	0.6
		31C	6.8
		35C	10.4
		39.3C	13.4

^a S-1 inoc: 1.1 X 10⁴ PFU/0.2 ml

^b parent inoc: 1.5 X 10⁴ PFU/0.2 ml

B. Effect of temperature on S-1 vaccine virus phenotype.

It was reported in the Annual Report, 1978, that temperature-dependent reversion was occurring with S-1 vaccine virus in FRhL cells. This was found by growing S-1 virus at 31C, 35C, and 37C in multiple culture flasks and examining the progeny from individual flasks for temperature resistant (tr) virus, i.e. virus that formed plaques at 39.3C. At 37C, 25% of flasks contained tr virus while all of those incubated at 31C produced temperature sensitive (ts) virus. This basic experiment was expanded to include a comparison between S-1 vaccine virus and S-1, p-19a (PGMK X 19) viruses. Both were grown at 31C, 35C, and 37C in FRhL cells. Table 4 lists the 7 day harvest titers of these viruses plaqued at 35C as well as the number of cell culture flasks containing virus which plaqued at 39.3C. The S-1 vaccine-inoculated flasks show an increase in the percentage of flasks with tr virus as a function of increased temperature. At 37C, 65% of these flasks contained virus in supernatant culture fluids that formed plaques at 39.3C, while at 31C, no tr virus was found

in any of the infected flasks. None of the S-1, p-19a virus-inoculated flasks at 31C, 35C, or 27C were found to contain virus, however virus titers were appreciably lower at all temperatures than virus from flasks inoculated with S-1 vaccine virus (Table 4).

Individual plaque isolates were made from plaques formed at 39.3C in four S-1 vaccine-inoculated flasks incubated originally at 37C. The plaque picks were inoculated into LLC-MK₂ flasks incubated at 35C and the supernatant fluids harvested after 14 days. Assays of the clone harvests from seven plaque picks listed in Table 5 revealed that 6 of 7 did not form plaques at 39.3C. This would indicate that these clones are not revertant and the one clone which plaqued at 39.3C appears to contain leaky virus.

C. Replication of vaccine virus in monkey peripheral blood leukocyte (PBL) cultures. Halstead and O'Rourke (1) demonstrated that replication of dengue virus occurred in monkey PBL cultures when the PBL's were from a dengue-immune monkey or when a non-immune cell donor was used and diluted dengue antibody was added to the culture system. Cells from a cynomolgus monkey, previously immunized with DEN-3, were inoculated with S-1 vaccine and S-1, p-19a viruses at a MOI of approximately 0.015. Human antisera with a DEN-2 neutralization titer of 1:80, previously shown to enhance dengue virus replication in human monocytes (Brandt *et al.*, in press), was added to cell culture medium at a final dilution of 1:500. Both dengue immune monkey PBL's and addition of diluted homologous antibody were used for maximal enhancement of growth of the S-1 viruses. On days 2, 3, and 4 post infection, triplicate vials were frozen at -70C and later assayed in LLC-MK₂ cells. Growth curve data listed in Table 6 indicate that the S-1 vaccine virus grows to higher titer than the S-1, p-19a virus in monkey PBL cultures.

Comparisons of S-1 vaccine and S-1, p-19a viruses were done in the two experiments reported on above to detect differences in these viruses. Additionally, it was found that the vaccine virus which is a product of 4 passages of S-1, p-19a virus in FRhL cells had changed immunogenically so that monkeys receiving the vaccine responded with lower titers of antibody than did monkeys which had previously been inoculated with S-1, p-19a virus (Eckels *et al.*, submitted for publication). Finally, a higher replicative capacity for the S-1 vaccine virus at 37C was found in comparative growth curves with the S-1, p-19a virus (Table 7).

Table 4. Emergence of tr S-1 virus as a function of increasing temperature.

Virus	Temp	No. cultures with tr virus/ total no. of cultures
S-1, p-19a	31C	0/21 ($9.9 \times 10^4 \pm 0.1$) ^a
	35C	0/23 ($8.6 \times 10^4 \pm 0.1$)
	37C	0/22 ($2.1 \times 10^4 \pm 0.2$)
S-1 vaccine	31C	0/23 ($3.4 \times 10^5 \pm 0.3$)
	35C	3/23 ($3.0 \times 10^5 \pm 0.2$)
	37C	15/23 ($1.8 \times 10^5 \pm 0.2$)

^a 7 day harvest mean titer \pm standard error of the mean.

Table 5. Plaque formation by clones of S-1 virus grown at 37C.

Clone	PFU/ml	
	35C	39.3C
1	1.5×10^5 ^a	< 5
2	7.5×10^5	< 5
3	6.0×10^5	< 5
4	4.3×10^5	< 5
5	3.7×10^5	< 5
6	2.0×10^5	< 5
7	8.0×10^5	6.0×10^2

^a Harvest of clones derived by plaque picking and amplification for 14 days in LLC-MK₂ cell cultures. Plaques selected for cloning were from a 39.3C assay of S-1 vaccine virus grown at 37C.

Table 6. Replication of S-1 vaccine and S-1, p-19a viruses in monkey peripheral blood leukocyte cultures.

Virus	Day	PFU/ml \pm Sm
S-1 vaccine	2	61 \pm 21
	3	205 \pm 45
	4	150 \pm 55
S-1, p-19a	2	20 \pm 2
	3	20 \pm 5
	4	3 \pm 2

Table 7. Comparative replication of S-1 vaccine and S-1, p-19a viruses in LLC-MK₂ cells.

Virus	Temp	Day	PFU/ml
S-1 vaccine	35C	2	2.2 X 10 ²
		3	1.5 X 10 ³
		4	2.6 X 10 ⁴
	37C	2	1.7 X 10 ²
		3	1.5 X 10 ³
		4	2.4 X 10 ⁴
S-1, p-19a	35C	2	5.0 X 10 ²
		3	7.5 X 10 ³
		4	4.8 X 10 ³
	37C	2	1.6 X 10 ²
		3	3.4 X 10 ³
		4	1.2 X 10 ³

In summary, the behavior of in vitro markers for S-1 vaccine virus which include higher replicative capacity at super-optimal temperature, increased leakiness, and increased capacity to infect monkey PBL cells does not extrapolate to observations found in vivo, i.e. monkey immunogenicity. There is no evidence that the S-1 vaccine virus replicates to higher levels in monkeys and, from antibody data, the reverse appears to be true. Details of these experiments may be found in Eckels et al. (submitted for publication).

D. Summary of dengue-2 vaccine human volunteer study no. 2. A study was carried out in 21 volunteers from Ft. Detrick, Md., and included 19 flavivirus nonimmunes and 2 with previous flavivirus experiences. The nonimmune volunteers received one of four dilutions of DEN-2 vaccine; undiluted (5), 10^{-1} (5), 10^{-2} (5) and 10^{-3} (4). The two flavivirus immunes received placebo inoculations.

Eight of the 19 vaccine recipients seroconverted and five had detectable viremias. The onset of viremia ranged from 9-13 days after vaccination and lasted 5-7 days. Virus isolates obtained from serum retained the growth characteristics of the vaccine virus. Antibody levels on day 30 were consistent with primary dengue infections. The frequency of seroconversion at each vaccine dilution did not correlate closely with virus dosage, so it was not possible to determine an ID_{50} for the vaccine virus. Instead, the results suggested that immunization of nonimmune volunteers is dependent on factors other than virus dosage.

None of the volunteers developed a local reaction at the injection site. However, a variety of symptomatic illnesses were reported by 11 of 21 volunteers during the 21 days following vaccination. Most of the complaints were attributed to incidental infections and trauma. Only 2 of 8 people who seroconverted to DEN-2 were symptomatic. One developed headache, myalgia, eye pain and rash consistent with clinical dengue; the other had an acute low back strain. One other viremic volunteers developed an asymptomatic rash. Leukopenia occurred in two people who seroconverted and one who did not. In total, only 1 of 8 people who seroconverted developed a dengue-like illness while 3 others had mild clinical changes associated with dengue.

Aedes aegypti mosquitoes fed on seven volunteers including three people who were viremic. Virus was isolated from 2 of 72 engorged mosquitoes that fed on one viremic volunteer. This experiment successfully demonstrated that viremic vaccinees are capable of infecting natural dengue vectors.

The results of this study suggest that the DEN-2 vaccine is sufficiently safe and immunogenic to merit additional testing.

Details of the human volunteer study may be found in the Annual Report, Department of Virus Diseases, WRAIR. The Department of Hazardous Microorganisms, the source of vaccine used in the trials, was involved in titration and characterization of virus from viremic blood and infected mosquitoes. Immunological responses were determined by HI, CF, and neutralization tests in this laboratory.

E. Radioimmunoassay of DEN-2 vaccinee sera.

1. Introduction.

The radioimmunoassay (RIA) has frequently been used in laboratories to detect molecules at the nanomole level or lower. RIA's were originally found useful for measuring blood levels of polypeptide hormones such as insulin. Later, RIA methodology was applied to the investigation of antibodies to microorganisms. These early RIA techniques measured the amount of radiolabelled antigen precipitated by specific antibody. More recent "solid phase" RIA procedures use either antigen or antibody attached to a solid surface such as a plastic tube or multi-well plate. Labelled primary or secondary antibodies are added to the test and the amount of bound antibody is measured. Solid phase RIA has become a useful immunological tool that does not rely on large amounts of intrinsically labelled antigens. Iodination procedures allow specific labelling of immunoglobulin molecules. Small volumes of reagents allow for many tests to be done in a short period of time. The sensitivity of the RIA for detecting small amounts of antigen and antibody is also well established.

As part of the DEN-2 immunization program, CF, HI and plaque reduction neutralization tests were performed to measure the humoral immune response of the human volunteers following DEN-2 vaccination. Because of the limitations of classical serology, a RIA was developed to detect antibody responses in vaccinees with low or undetectable antibody measured by standard tests. In addition to IgG responses, volunteer sera were assayed for the presence of IgM and IgA immunoglobulins. Finally, the appearance of RIA antibodies was compared with the increase in titer found for antibodies measured by CF, HI and neutralization tests.

2. Materials and Methods.

a). Growth of virus. One ml of DEN-2 strain PR-159 (GM-6, FRhL-3) virus was inoculated into 32 oz. bottles containing monolayers of LLC-MK₂ cells or a line of Aedes albopictus (C636)

cells obtained from Dr. Joel Dalrymple, Department of Virus Diseases, WRAIR. LLC-MK₂ cells were maintained in Medium 199 containing 2% fetal bovine serum, 0.1% penicillin/streptomycin, 0.05% fungizone and grown at 35 C. The A. albopictus cell line was maintained with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% glutamine, 1% non-essential amino acids, 0.1% penicillin/streptomycin and 0.05% fungizone, and incubated at 28 C. Infected cell cultures were harvested repeatedly at 2-5 day intervals until extensive cytopathic effects were observed in the cell monolayers. Virus harvests were taken for a period of 17 days in the LLC-MK₂ cells and for 42 days from the A. albopictus cell cultures.

b). Virion purification. Virus harvests were initially clarified by centrifugation at 2,000 rpm for 20 minutes. Polyethylene glycol (PEG) precipitation was performed by adding 2.2 gms of NaCl per 100 ml of supernatant fluid and 7.0 gms of PEG 6000 and mixed by a magnetic stirrer in an ice bath for 4 hr. The precipitate was centrifuged in a GSA rotor for 50 minutes at 9,500 rpm and the pellet was resuspended in Tris saline-EDTA buffer, pH 7.8, in 0.01 of the original volume. Four ml of the PEG-precipitated virus concentrate was layered on an 8 ml linear potassium tartrate-glycerol gradient and centrifuged in a SW41 rotor for 16 hr at 40,000 rpm. After centrifugation, twenty-four 0.5 ml fractions were collected from each gradient and screened for HA and RIA activity. Those fractions with the highest HA and RIA activity were pooled and frozen at -70 C. An optimal concentration of antigen was determined by checkerboard titration against mouse hyperimmune ascitic fluid.

c). RIA procedure. The following procedure was developed by Dr. Joel Dalrymple and used by our laboratory for RIA of human sera. A drop (25 μ l) of antigen diluted in Dulbecco's phosphate buffered saline was added to soft microtiter plates (Cooke Engineering #220-24) and was air dried overnight at 35 C. The microtiter plates were washed 4 times with 50 μ l of "filler" which consisted of Dulbecco's PBS containing 20% calf serum, 0.2% sodium azide and 0.01% phenol red. After the microtiter plates were washed, 100 μ l of filler was added to the wells and incubated for 1 hr at 35 C. The filler was aspirated and the wells refilled with 25 μ l of test sera and incubated overnight at room temperature. The following day the wash procedure described above was repeated. ¹²⁵I antiglobulin or ¹²⁵I protein A was added to the wells in a volume of 25 μ l and incubated overnight at room temperature. The wash procedure was repeated again as described above. The microtiter plates were air dried, and the wells cut from the plate and placed individually into counting tubes to be counted for 1 minute intervals in a Nuclear of Chicago gamma counter.

3. Results.

a). Effect of antigen in RIA. Preliminary RIA experiments were done using DEN-2, New Guinea C virion and cell antigens to measure antibody in human volunteer sera (Dr. Joel Dalrymple, personal communication). DEN-2 antibodies were found in 6/17 (38%) vaccinees tested using extracted, virus infected CRC cell membrane antigen. This percentage was increased to 63% using virion antigens. In order to increase the sensitivity of the RIA for screening vaccinee antibody, the vaccine strain (PR-159) virus was used. RIA's employing vaccine strain virions were able to detect antibody in several previously tested "non-responders". The percentage of vaccinees with detectable DEN-2 antibodies was raised to 87% (13/17). As was shown previously in neutralization tests, the antigenic difference between DEN-2, New Guinea C and PR-159 virus was also evident by RIA.

b). Appearance of RIA antibodies. Sera from eleven volunteers were studied by the RIA for the temporal appearance of antibodies following vaccination. Included were yellow fever "immunes" who received the DEN-2 vaccine (WHB, WLO, FHT); those with no previous flavivirus experience who received the vaccine and demonstrated the presence of HI, CF or neutralizing antibodies (GFM, KMM, JSR, JFS and KLT); and the final 2 volunteers (SEG and SDF) who were also flavivirus "virgins" when the vaccine was administered and did not show evidence of antibody stimulation following vaccination.

Human volunteer sera from pre-vaccination (day 0) to 1 year post vaccination were serially diluted in 0.5 log increments starting at a 1:10 dilution. IgG levels were measured either by ^{125}I -radiolabelled goat anti-human IgG or ^{125}I -radiolabelled protein A. IgM and IgA were measured using ^{125}I -radiolabelled goat anti-human specific immunoglobulins. An RIA endpoint titer was expressed as a dilution of serum that gave 1.5 times the number of counts found for the control (day 0) serum. Allowances were taken for prozone phenomena observed with the lower dilutions of sera resulting in counts less than 1.5 times above control (day 0) serum. This effect at the lower dilutions of sera is demonstrated in Figure 1 where the IgM RIA for KLT shows a prozone phenomenon at serum dilutions of 1:10 and 1:30.

Table 8 lists IgG and IgM antibody responses for 9 volunteers using "single point" determinations, i.e., each serum dilution was represented by one sample. Immunoglobulin G was measured by both labelled protein A and labelled anti-human IgG. The data presented in Table 8 compares IgG titers by both

methods. Protein A was slightly more sensitive in measuring IgG levels than anti-human IgG. Most tests using protein A had less binding of label to the control serum allowing for a slight increase in the sensitivity of the test. However, serum end-point titers found by the two methods differed by as much as 1.5 logs. Protein A detected 36 antibody-positive days compared to 34 days found by anti-human IgG. Anti-human IgG was in 83% agreement with protein A in detecting antibody-positive days.

Immunoglobulin G responses were generally detected by day 21. The earliest detectable IgG was observed on day 10 for volunteers WHB, SEG, JSR and RLS. IgG levels usually peaked between days 21 and 60. In most instances, IgG levels persisted with slight titer reductions in 6 month post-vaccination sera. Exceptions were volunteers GFM and RLS, who demonstrated an increase in 6 month IgG titers.

Generally, detection of IgM coincided with the appearance of IgG on day 21 post-vaccination. Earlier IgM responses were observed and can be found in Table 8. IgM was detected on day 4 for vaccinee SEG and on day 7 for volunteers RLS and WHB. In contrast to peak IgG titers, peak IgM titers were usually found to be 1 to 2 logs lower. Volunteers SEG and JFS were two exceptions showing peak IgM levels titering a log higher than peak IgG levels. In the majority of volunteers, IgM was not found in 6 month sera. Only GFM had detectable IgM after 6 months.

To decrease the variation introduced by single point tests, each serum was tested in triplicate and a mean was calculated. Also, to adjust for individual serum "stickiness", the test serum was added to RIA plates containing no antigen. Bound counts were considered non-specific and subtracted from appropriate test counts. Table 9 lists the results of IgG, IgM and IgA levels for 7 vaccinees assayed in triplicate in the RIA. Volunteer KLT's Ig levels assayed this way are graphed in Figure 1. Graphs showing Ig levels using triplicate point determinations were more consistent and interpretable than graphs where single point determinations were used. Table 9 lists Ig responses for volunteers taken at three intervals after vaccination. These intervals were chosen to detect and evaluate early, optimal and late antibody development.

In comparing IgG and IgM levels between Tables 8 and 9, there is an increased sensitivity in detecting antibody using

Table 8. Human response to DEN-2 vaccine measured by RIA (single pt determination).

Vaccinee	Interval post vaccine	RIA		
		Protein A	anti-IgG	anti-IgM
WHB	7 days	- ^a	-	10
	10	10 ^b	300	-
	14	-	- ^c	-
	17	100	*	-
	21	≥10,000	≥30,000	-
	30	≥10,000	≥30,000	*
	60	≥10,000	≥30,000	*
	6 months	≥3,000	≥30,000	-
	12	≥3,000	≥30,000	-
WLO	4 days	-	-	-
	7	-	-	-
	10	-	-	-
	14	3,000	*	-
	21	≥30,000	≥30,000	30
	60	≥30,000	≥30,000	*
	6 months	3,000	≥30,000	-
	12	*	3,000	-
SDF	4 days	-	-	-
	7	-	-	-
	10	-	-	-
	14	-	-	30
	17	-	-	*
	21	100	100	*
	45	10	100	*
	60	-	300	*
	6 months	-	-	-

^a indicates titer <10.

^b Serum titers are recorded as the reciprocal of the serum dilution that bound labelled protein A or Ig at 1.5 X above control (day 0) serum.

^c indicates evidence of positive reaction but is not 1.5 X above control (day 0) serum.

Table 8. (CONTINUED) Human response to DEN-2 vaccine measured by RIA (single pt determination).

Vaccinee	Interval post vaccine	RIA		
		Protein A	anti-IgG	anti-IgM
SEG	4 days	- ^a	* ^c	300
	7	* ^b	*	*
	10	10 ^b	300	3,000
	14	*	-	*
	17	10	*	1,000
	21	-	-	-
	30	-	-	10
	45	10	300	1,000
	60	-	-	-
GFM	4 days	-	-	-
	7	-	-	-
	10	-	-	-
	14	-	-	-
	17	-	-	-
	21	10	10	100
	30	*	*	100
	45	3,000	1,000	100
	60	≥30,000	10,000	100
	6 months	≥30,000	≥30,000	300
JSR	4 days	-	-	-
	7	-	-	-
	10	-	10	10
	14	-	-	-
	17	-	*	*
	21	*	*	30
	30	100	100	100
	45	1,000	1,000	10,000

^a indicates titer < 10.

^b Serum titers are recorded as the reciprocal of the serum dilution that bound labelled protein A or Ig at 1.5 X above control (day 0) serum.

^c indicates evidence of positive reaction but is not 1.5 X above control (day 0) serum.

Table 8. (CONTINUED) Human response to DEN-2 vaccine measured by RIA (single pt determination).

Vaccinee	Interval post vaccine	RIA		
		Protein A	anti-IgG	anti-IgM
RLS	4 days	- ^a	* ^c	-
	7	-	*	10
	10	100 ^b	1,000	100
	14	*	*	300
	17	1,000	1,000	300
	21	1,000	1,000	3,000
	30	10,000	3,000	10,000
	60	≥30,000	≥30,000	3,000
JFS	4 days	-	-	-
	7	-	-	-
	10	-	-	-
	14	-	-	-
	17	-	*	-
	21	-	-	*
	30	3,000	*	10,000
	45	10,000	3,000	3,000
	60	10,000	3,000	10,000
	6 months	300	*	-
KLT	7 days	-	-	-
	10	-	-	-
	14	-	-	-
	17	-	-	-
	21	10,000	1,000	1,000
	30	3,000	300	300
	45	≥30,000	≥30,000	*
	60	3,000	300	-
	6 months	10,000	30	-

^a indicates titer < 10.

^b Serum titers are recorded as the reciprocal of the serum dilution that bound labelled protein A or Ig at 1.5 X above control (day 0) serum.

^c indicates evidence of positive reaction but is not 1.5 X above control (day 0) serum.

Table 9. Human response to DEN-2 vaccine measured by RIA
(triplicate pt determination)

Vaccinee	Interval post vaccine	RIA		
		anti-IgG	anti-IgM	anti-IgA
SDF	day 17	300	30	ND
	day 45	30	30	
	6 months	30	30	
KMM	day 21	≥30,000	300	ND
	day 30	≥30,000	100	
	4 months	≥30,000	-	
JFS	day 17	-	-	ND
	day 30	30	3,000	
	6 months	300	-	
KLT	day 17	100	-	-
	day 30	1,000	100	3,000
	6 months	300	-	300
WHB	day 17	30	100	ND
	day 30	≥30,000	3,000	
	6 months	≥30,000	3,000	
WLO	day 21	≥30,000	30	ND
	day 60	≥30,000	-	
	6 months	10	-	
FHT	day 17	100	-	-
	day 30	≥30,000	3,000	3,000
	6 months	≥30,000	-	1,000

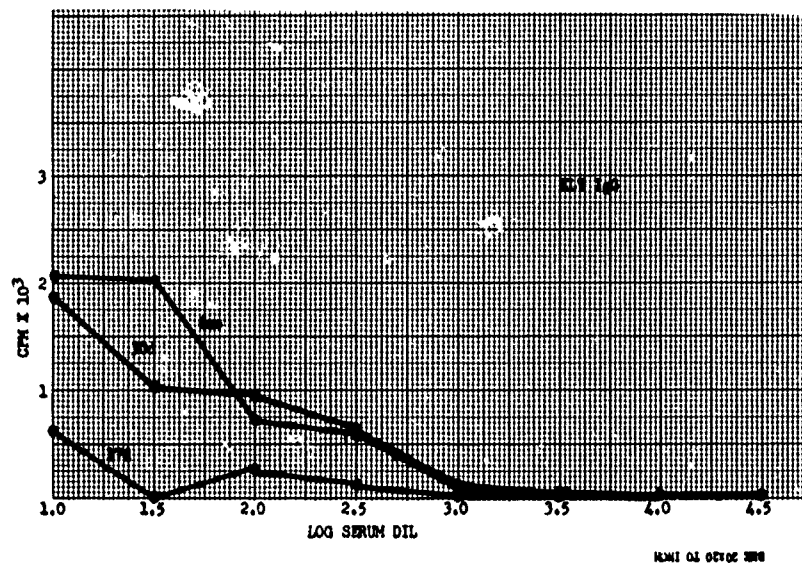


Figure 1. (a) Ig response for human volunteer KLT measuring IgG.

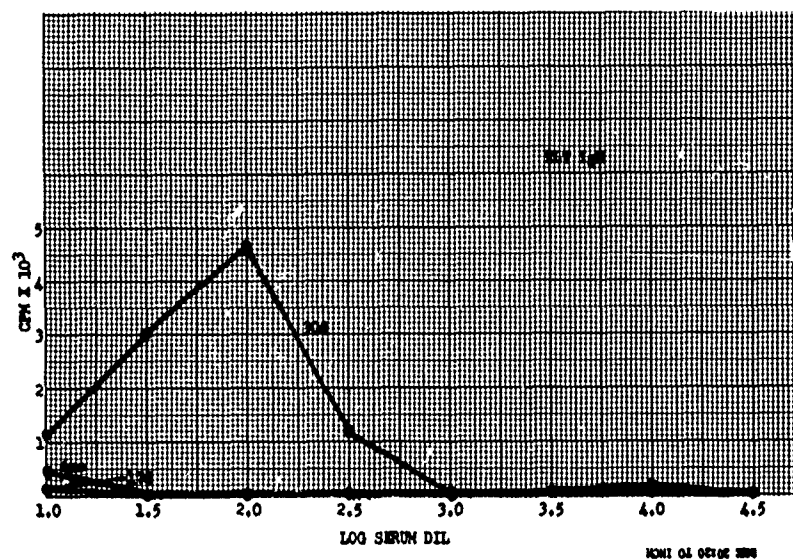


Figure 1. (b) Ig response for human volunteer
KLT measuring IgM.

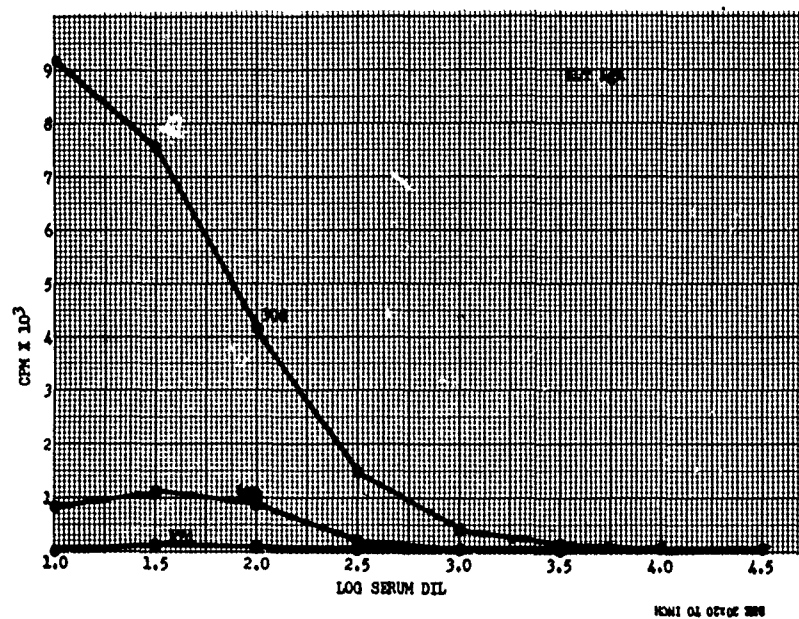


Figure 1. (c) Ig response for human volunteer KLT measuring IgA.

triplicate sampling. Table 9 lists 4 more additional IgG-positive days. Volunteers SDF, JFS and KLT were positive on day 17 and IgG was evident in SDF's 6 month serum. Table 9 further notes the detection of 5 more IgM positive days not listed in Table 8. These IgM responses were measured for SDF and WHE. Otherwise, both Tables 8 and 9 expressed similar IgG and IgM patterns. RIA measurement of 2 vaccinee's IgA response can be found in Table 9. Both the yellow fever "immune" (FHT) and flavivirus "virgin" (KLT) gave analogous IgA antibody responses. Both volunteers showed no evidence of detectable IgA on day 17. Similarly, both IgA titers peaked at day 30 after immunization, followed by a reduced but still persistent level of IgA at 6 month's post vaccination.

4. Summary.

The main objectives of this study were clearly attained. The first 3 classes of immunoglobulins, IgG, IgM and IgA were definitely demonstrated in both yellow fever "immunes" and flavivirus "virgins". Secondly, IgG and IgM antibodies were detected by RIA in previously classified "non-responder" volunteers. The advantages of the RIA are many. These include 1) μ l amounts of reagents; 2) high degree of sensitivity for detection of viral antibody; 3) versatility to measure different classes of immunoglobulins. Future studies should include: 1) improved methodology for test procedures and interpretation of positive endpoint titers; 2) RIA's to measure the specificity and cross reactivity of sera for other DEN serotypes and yellow fever antigens.

II. Dengue-3 Vaccine Progress.

A. Preparation of a production seed for the C-5 clone of DEN-3. Previous Annual Reports have documented isolation and passage of a clone (C-5) of DEN-3, CH53489 to a master seed prepared in FRhL cells. Following procedures used for the master seed, a production seed was prepared using FRhL roller flasks inoculated with C-5 master seed virus. A final harvest was taken on day 21 with several media changes occurring up to this time. Plaque assays on individual flasks done immediately on day of harvest revealed that all usable flasks contained virus that plaqued at 39.3 C. EOP values (39.3 C/35 C PFU ratio) were high compared to values from other seeds made previously. The EOP range was 0.034 to 0.15 for 10 flasks of the production seed while a 10th passage C-5 seed had an EOP of ≤ 0.001 . Further examination of the pooled harvest revealed

growth of the C-5 seed at 38.5 C and also a high degree of replication in suckling mouse brain (2.1×10^3 PFU/0.2 ml by day 7). Mixed plaque size at 35 C was also quite evident. This seed could not be used for vaccine production.

B. Preparation of master seeds for C-5 sublines. From the results of the production seed and some evidence of mixed plaque size and tr virus in the master seed, it was agreed upon to use the 14th passage of the C-5 sublines to prepare 7 small batches of master seed. Each subline was inoculated into FRhL roller flasks and harvests taken on day 12. Results of plaque assays of the harvests are listed in Table 10. Some degree of heterogeneity was evident in plaques seen at 35 C and low titers of virus were seen in 39.3 C assays. A day 21 harvest revealed some increase in tr virus that plaqued at 39.3 C.

Each harvest was also assayed in suckling mice by intracerebral inoculation and brain pool titers are listed in Table 11. Two mice inoculated for each subline were sacrificed daily for 10 days and 20% brain homogenates were prepared for plaque assay. All virus recovered from the homogenates was small plaque. Titters were comparable to those found for earlier passages of C-5. The parent virus (FRhL-6) in similar assays has reached titers of $\geq 10^4$ PFU/0.2 ml. By this marker, the C-5 sublines looked attenuated.

To test for homogeneity of the C-5 subline master seeds and also to test for leakiness and reversion in these seeds, individual clones were isolated from 3 of the subline seeds. Plaques were picked from assays done at 35 C and 39.3 C and inoculated into LLC-MK₂ cell culture. A 14 day harvest was taken for each clone and assayed at 35 C and 39.3 C. Table 12 lists the characteristics of 24 clones derived from 3 sublines. Four clones were also derived from the parent (FRhL-6) virus and included in the table. From the 35 C plaque picks (12) of the 3 sublines, 8 contained tr virus, i.e. virus that plaqued at 39.3 C. Six of these 8 clones also had mixed plaque morphology. Of those clones derived by picking plaques from a 39.3 C assay, 9 of 12 contained tr virus and all 9 had mixed plaque morphology.

The high degree of heterogeneity in these seeds made them unusable. The emergence of tr virus to high titers in previous master and production seeds was most likely linked to this heterogeneity. The cloning characterization as described above had to be used to substantiate the presence of tr virus popula-

Table 10. Master seed harvests (FRhL-16b, days 12 and 21)
for the 7 sublimes of the C-5 clone.

Subline	day 12		day 21	
	PFU/0.2 ml		PFU/0.2 ml	
	35 C	39.3 C	35 C	39.3 C
1	1.3×10^4	1.3×10^1	2.3×10^3	1.2×10^1
2	1.3×10^4	1.1×10^1	1.3×10^3	6
3	8.2×10^3	1.5×10^1	4.8×10^2	3
4	6.7×10^3	9	1.4×10^3	3
5	1.4×10^4	3	6.1×10^2	8
6	1.0×10^4	7	3.8×10^2	1.2×10^1
7	1.5×10^4	2.7×10^1	3.8×10^2	5

Table 11. Master seed harvests (FRhL-16b, day 12) in suckling mice.

Subline	Brain homogenate	Day of max titer
	PFU/0.2 ml	
1	25	7
2	4	5
3	7	6
4	4	7
5	10	5
6	6	10
7	4	6

Table 12. Plaque characteristics of clones from master seed (C-5) sublines 1, 5, and 6.

Plaque Pick#	Subline	Plaque Morph	14 Day SOP Yield, p-18			Plaque Morph
			35C	39.3C	EOP	
1	1	L (35C)	2.4×10^3	7	0.003	Mix
2		S	3.1×10^3	1.8×10^1	0.006	Mix
3		S	2.9×10^3	0	< 0.0003	S
4		L	1.4×10^3	5.7×10^2	0.04	Mix
5	1	S (39.3C)	1.0×10^3	8.8×10^1	0.09	Mix
6		S	5.2×10^2	0	0.002	S
7		S	4.5×10^2	4.7×10^2	0.1	L
8		S	6.5×10^2	5	0.008	Mix
9	5	S (35C)	2.6×10^3	4	0.002	S
10		S	3.3×10^3	2.1×10^1	0.006	Mix
11		S	2.2×10^3	0	< 0.0005	S
12		S	1.3×10^3	3.0×10^1	0.02	Mix
13	5	S (39.3C)	1.9×10^3	5.6×10^1	0.03	L
14		S	4.4×10^3	7.1×10^2	0.16	L
15		S	4.1×10^3	3.9×10^1	0.1	L
16		S	4.0×10^3	3.7×10^1	0.009	Mix
17	6	L (35C)	2.8×10^3	4.8×10^1	0.02	Mix
18		L	1.9×10^3	0	< 0.0005	S
19		S	5.6×10^2	2	0.004	S
20		S	5.9×10^2	0	< 0.002	S
21	6	S (39.3C)	4.3×10^2	0	< 0.002	Mix
22		S	3.6×10^2	0	< 0.003	Mix
23		S	2.5×10^2	2.7×10^2	1.1	L
24		S	6.4×10^2	2.5×10^2	0.4	L
25	PAR, FRhL-5	S (35C)	1.5×10^4	3.4×10^3	0.2	Mix
26		L	1.2×10^3	2.0×10^3	0.2	Mix
27		L	3.2×10^3	1.0×10^3	0.3	Mix
28		L	7.6×10^3	1.3×10^3	0.2	Mix

tions. Since the virus had to be grown for 14 days, there is also the possibility of reversion occurring during passage. This would be an extremely high rate of reversion that would be unexpected for this virus.

Heterogeneity in the master seed would indicate: 1) emergence of revertants upon passage or 2) unsuccessful cloning of the original isolate. Since terminal dilution techniques had to be used for the series of clonings done at passages 11 to 13, there is a good chance that individual, genetically homogeneous virus populations were not derived by these procedures. Also, amplification of the original C-5 isolate at passages 9 and 10 for "adaptation" to FRhL cells may also have introduced unwanted, tr virus. One or both of these hypotheses to explain the heterogeneity of the C-5 seeds is valid.

C. New line of C-5 passages. The C-5 clone, originally purified by terminal dilution cloning of the parent (FRhL-6) virus, was prepared in a seed at the 8th passage level. Amplification at passages 9 and 10 were done for adaptation purposes as described above. Another line of passages was made previously to this which consisted of terminal dilution cloning steps at passages 9 and 10 and a seed preparation at passage 11 in FRhL cells. The passage designated FRhL-11b was used for initiation of a FRhL-12b passage. A 12 and 14 day harvest of the C-5 clone at this passage level was characterized by cloning as described above. A series of 30 clones from a 35 C plaque assay were examined, 14 from the day 12 harvest and 16 from the day 14 harvest. Only one clone, from the day 12 harvest, contained tr virus.

Another passage (FRhL-13b) of C-5 was made in FRhL rollers and a 12 day harvest was again characterized for tr virus clones. Out of a series of 39 clones, 23 contained virus that plaqued at 39.3 C. Seventeen of the 23 tr clones also had mixed plaque morphology consisting of small, medium, and large plaques. Again, emergence of tr virus or reversion from ts to tr virus appeared in a C-5 seed. Attempts to adequately clone this virus and to find more stable subpopulations are underway. These passages will be done by plaquing in PGMK cells.

D. Freeze-drying of a C-5 seed. The C-5 clone, FRhL-12b was used for defining conditions of freeze-drying for maximal recovery of virus. Previously, FBS or human serum albumin (HSA) have been used as stabilizers for freezing and freeze-drying. Preliminary data with C-5 seeds indicated that virus

was lost when frozen in FBS concentrations to 50%. Sucrose has been used in some bacterial seeds that are freeze-dried to maintain viability upon rehydration (S. Berman, personal communication). Sucrose combined with FBS or HSA, FBS or HSA alone, and absence of stabilizer were examined for freezing and freeze-drying of the C-5, FRhL-12b seed. Table 13 lists results in terms of percent loss of virus on freezing or freeze-drying using a fresh titrated sample as a standard of comparison. Addition of sucrose appears to be useful in stabilizing C-5 virus after freeze-drying in HSA and to a lesser extent in FBS.

Table 13. Stabilization of C-5 virus during freezing and freeze-drying.

Stabilizer	Treatment	PFU/0.2 ml	% loss
none	control	3 4.7 X 10 ³	-
	freeze/thaw	1.1 X 10 ¹	77
	freeze-dry + rehydrated	2.0 X 10 ¹	99
FBS (50%)	control	3 3.3 X 10 ³	-
	freeze/thaw	2.8 X 10 ³	15
	freeze-dry + rehydrated	3.0 X 10 ³	9
FBS (50%) + sucrose (7.5%)	control	3 3.4 X 10 ³	-
	freeze/thaw	2.7 X 10 ³	21
	freeze-dry + rehydrated	1.0 X 10 ⁴	0
HSA (2.5%)	control	3 5.3 X 10 ³	-
	freeze/thaw	1.5 X 10 ²	72
	freeze-dry + rehydrated	4.9 X 10	91
HSA (2.5%) + sucrose (7.5%)	control	3 5.6 X 10 ³	-
	freeze/thaw	7.0 X 10 ⁴	0
	freeze-dry + rehydrated	1.4 X 10	0

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 014 Characteristics of attenuated dengue viruses

Literature Cited.

References:

1. Halstead, S.B., and O'Rourke, E.J. Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. J. Exp. Med. 146: 201-217, 1977.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD FORM 1498-1	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8A. DESIG INSTR ^a	8B. SPECIFIC DATA CONTRACTOR ACCESS ^a	9. LEVEL OF SUM A. WISE V-ST
78 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62770A	3M762770A802	00	015			
B. CONTRIBUTING							
C. CONTRIBUTING	CARDS 114F						
11. TITLE (Precede with Security Classification Code) ^a							
(U) Chemotherapy and Chemoprophylaxis of Schistosomiasis							
12. SCIENTIFIC AND TECHNOLOGICAL AREA ^a							
002600 Biology 012600 Pharmacology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
7810		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA				PRECEDING			
B. NUMBER: NA				FISCAL YEAR		125	
C. TYPE: NA				CURRENT YEAR		129	
D. KIND OF AWARD: NA				F. CUM. AMT.			
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER.			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME: Greene, Lyford K., CPT, MSC, Ph.D.			
				NAME: Grenan, Marie M. GS-12			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Animal Models; (U) Schistosomiasis; (U) Drug Development; (U) Antiparasitic							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To find new drugs with chemoprophylactic or chemotherapeutic activity against Schistosoma mansoni, which can be used by military personnel to prevent or treat the disease in endemic areas.							
24. (U) Compounds previously shown to be active in screening tests will be reexamined to determine their optimum prophylactic and/or therapeutic treatment regimens in the mouse and/or subhuman primate models. Prophylactic agents for either topical application or systemic administration are being developed. Available chemical analogues will be tested and efforts will be made to determine the relationships between chemical structures, modes of antiparasitic action and modes of toxicity. Analogues with increased therapeutic efficacies and decreased host toxicities will be identified, and where justified, submitted for preclinical studies.							
25. (U) During this fiscal year, sixty-eight compounds were screened topically in the secondary prophylactic test using ICR mice. Of these, thirty-four were active when tested without post-application water wash and eighteen of these had activity even after a 1/2 hour running-water wash. The most active structural classes were the 4-aminoquinolines (13 active of 21 tested), the 8-aminoquinolines (3/3), and the biphenyls (5/14) and there were 10 active chemicals of miscellaneous structures (of 24 tested). A selected 4-aminoquinoline and hexachlorophene each inhibited cercarial penetration <u>per se</u> . A nitro-biphenyl isothiocyanate was, in contrast shown to have a delayed antischistosomal effect. For technical report see Walter Reed Army Institute of Research Annual Report, 1 Oct 78 - 30 Sept 79.							

^aAvailable to contractors upon originator's approval

1683

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PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

267

PROJECT 3M762770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 015 Chemotherapy and Chemoprophylaxis of Schistosomiasis

Investigators

Principal: CPT Lyford K. Greene, MSC

Associate: Marie M. Grenan

COL David E. Davidson, Jr., VC

1. Description

Schistosomiasis is a debilitating group of diseases resulting from intra-vascular egg deposition by the adult worms of Schistosoma mansoni, S. japonicum, S. mekongi or S. haematobium. Species of human schistosomes occur in freshwater environments in areas in the Caribbean, northern South America, Africa, the Middle East and the Far East. U.S. military forces deployed in any of these areas would be exposed at great risk if safe, effective prophylactic antischistosomal drugs were not available and, to date, there is no drug which has been approved for prophylactic use against any human schistosome.

The Schistosomiasis Section has been screening and evaluating chemicals against the Puerto Rico strain of S. mansoni cercariae to assist in the development of effective prophylactic antischistosomal drugs. Test compounds were applied to tails of mice and each animal was subsequently individually exposed to schistosome infection by immersion of the tail in water containing approximately 100 cercariae. Parameters of activity are the number of cercariae remaining in the exposure fluid and the number of adult worms recovered by perfusion on Day 49 post exposure.

In addition, the Schistosomiasis Section coordinates and supports screening operations conducted at the U.S. Army Medical Research Unit at the University of Brasilia, Brazil. Candidate drugs are tested for prophylactic activity in the Primary Mortality Test (drugs administered two days after exposure to cercariae) and for curative activity in the Primary Curative Test (drugs administered on Days 33-37 after infection).

2. Progress

During the past year at the University of Brasilia, 410 compounds were tested for prophylactic activity against S. man-

soni (Paulista strain). Of these, 132 were toxic and 19 were active. Of 343 compounds tested in the curative screen, 54 were toxic and 24 had activity.

The Schistosome Section examined 68 compounds for topical prophylactic activities. Results are summarized in Table 1. Thirty-four of the compounds were active when tested without running-water washes before exposure. Eighteen retained their activities even when application was followed by a one-half hour running-water wash. The most active structural classes were the 4-aminoquinolines, the 8-aminoquinolines and the biphenyls. There were 10 active compounds of miscellaneous structures.

Some of the 4-aminoquinolines were active at concentrations below 0.625% (w/v). A 0.312% solution of WR 93,156 provided complete protection with or without a one-half hour water wash. A 0.625% solution significantly inhibited cercarial penetration even when the application was followed by a three-hour running water wash.

WR 234,927 and WR 234,928, related chemicals of the biphenyl group, possessed high prophylactic activities when the interval between application and cercarial exposure was limited to one day. Recoveries of cercariae from the exposure vessels at the end of the exposure period were only slightly elevated over those of solvent-treated control groups, but less than two percent of the exposure dosage was found in liver as adult worms. It is apparent that these compounds afforded protection despite their failure to inhibit cercarial penetration of skin. On Day One after exposure, approximately the same concentrations of schistosomula were present in samples of tail skin of both WR 234,927-treated and solvent-treated mice. The migration of the schistosomula from the skin by Day Five appeared to be similar in both groups. Hence, it is concluded that the skin was not the site of activity of WR 234,927. Investigations to establish the site(s) of worm death resulting from treatment with this chemical are being continued.

The most active tested biphenyl was hexachlorophene which appeared to prevent cercarial penetration. In Table 2, it is indicated that hexachlorophene provided some protection even when the application was followed by a five-hour water wash. Useful protection persisted through washes as long as three hours. (Histological examination of skin of tails exposed to S. mansoni cercariae confirmed that extremely few cercariae penetrated into 1.25% (w/v) hexachlorophene-treated tails when the interval between chemical application and cercarial exposure is

kept short.) In Table 3, it is seen that useful protection continued to three days under dry conditions and there was some protection at six and seven days. Hexachlorophene apparently is rapidly cercaricidal and some of the observed protection may have been due to hexachlorophene which had been leached from the treated skin into the exposure fluid. To test this possibility, tails of mice with various pretreatments were immersed in water in exposure tubes for one hour. The treated mice were removed, cercariae were added to the tubes and previously untreated mice were exposed. The results, in Table 4, demonstrates that hexachlorophene was leached from treated skin into the exposure fluids even when its application had been followed by a five-hour running water wash.

Important observations made during this past year's research include the following:

1. Some effective topically-applied compounds inhibit cercarial penetration but others apparently have a delayed effect resulting in eventual worm death in the skin or in deeper host tissues.
2. Toxic amounts of some active chemicals still diffuse from the skin into the exposure fluid even when post-application water washes of several hours are employed.

Table 1. Classes of Compounds Tested Topically for Anti-Schistosomal Activity and Numbers of Members Showing Activity.

Class	Number Tested	Number Active	Number Active After Water Wash*
Acridines	1	1	0
Acridinophenones	1	0	0
4-Aminoquinolines	21	13	6
8-Aminoquinolines	3	3	3
Anthracene	1	0	0
Biphenyls	14	5	4
Wood Extracts	3	2	0
Miscellaneous	24	10	5
TOTALS	68	34	18

*Wash of 1/2 hour duration.

Table 2. Percent Cercarial Exposure Dosage Recoverable as Unpenetrating Cercariae or Day-49 Adult Worms from Mice Whose Tails were Pre-treated with 1.25% (w/v) Hexachlorophene and a Subsequent Water Wash of Various Durations.

Duration of Water Wash	Percent Unpenetrating Cercariae Recovered*	Percent Adult Worms Recovered* on Day 49*
one hour	58.9 ± 11.2** (10)	0.0*** (10)
two hours	60.4 ± 11.6** (10)	0.3*** (10)
three hours	42.3 ± 10.2** (10)	2.8 ± 2.1*** (10)
four hours	38.4 ± 11.8** (09)	10.7 ± 6.7*** (10)
five hours	17.1 ± 8.8** (10)	19.7 ± 9.8*** (10)

*Mean ± 2 Standard Errors

**Corresponding values for the methanol controls were less than 2.3%.

***Corresponding values for the methanol controls were 35 to 42%.
(n) Sample size

Table 3. Percent Cercarial Dosage Recoverable as Unpenetrating Cercariae and as Adult Worms from Mice Exposed to Cercariae at Different Times Post-Hexachlorophene-Treatment or Post-Methanol-Treatment.

Treatment	Unpenetrating Cercariae Recovered	Adult Worms Recovered On Day 49
1.25% (w/v) hexachlorophene in methanol three days pre-exposure	61.7 \pm 9.8* (09)	0.0 \pm 0.0* (10)
methanol three days pre-exposure	1.1 \pm 1.0 (10)	39.4 \pm 8.5 (10)
1.25% (w/v) hexachlorophene in methanol six days pre-exposure	28.1 \pm 8.4 (10)	11.2 \pm 9.7 (09)
methanol six days pre-exposure	0.9 \pm 0.6 (10)	29.4 \pm 4.5 (09)
1.25% (w/v) hexachlorophene in methanol	18.6 \pm 11.4 (08)	10.6 \pm 11.2 (08)
methanol seven days pre-exposure	2.4 \pm 1.6 (10)	31.7 \pm 5.8 (10)

*Mean \pm 2 Standard Errors
(n) Sample size

Table 4. Cercaricidal Effects of Hexachlorophene Leaching into Cercarial Exposure Media

Exposure Medium Pre-Conditioned by One Hour Immersion of Mouse Tail Treated with the following:	Percent Cercarial Dosage Recoverable as Unpenetrating Cercariae Following Exposure to Untreated Mice	Percent Cercarial Dosage Recoverable as Day-49 Adult Worms Following Exposure of Untreated Mice
1.25% (w/v) methanolic hexachlorophene	71.5 ± 5.8* (24)	0.05 ± 0.10* (25)
1.25% (w/v) methanolic hexachlorophene + 3 hour water wash	69.7 ± 5.6 (15)	0.05 ± 0.1 (14)
1.25% (w/v) methanolic hexachlorophene + 5 hour water wash	71.9 ± 11.2** (15)	5.8 ± 8.0** (15)
methanol	0.9 ± 0.4 (15)	54.5 ± 4.4 (15)
methanol + 3 hour water wash	2.7 ± 1.6 (14)	51.3 ± 5.8 (13)
methanol + 5 hour water wash	0.7 ± 0.6 (15)	57.2 ± 5.8 (11)

* Mean ± 2 Standard Errors; (Sample Size)

**Includes an apparently untreated animal

Project 3M162770A803

DRUG DEVELOPMENT

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a		2. DATE OF SUMMARY ^a		REPORT CONTROL SYMBOL		
				DA OB 6495		79 10 01		DD FORM 1498A (AR) 836		
3. DATE PREVIOUS ^a		4. KIND OF SUMMARY		5. SUMMARY SCTY ^a		6. WORK SECURITY ^a		7. REGRADING ^a		
78 10 01		D. Change		U		U		NA		
								8. DISB INSTR ^a		
								NL		
								9. SPECIFIC DATA: CONTRACTOR ACCESS		
								<input type="checkbox"/> YES <input type="checkbox"/> NO		
								10. LEVEL OF SUM		
								A. WORK UNIT		
10. NO / CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER		
6. PRIMARY		62770A		3M162770A803		00		084		
7. CONTRIBUTING										
8. CONTRIBUTING		CARDS 114F								
11. TITLE (Precede with Security Classification Code) ^a										
(U) Synthesis of Antiparasitic Drugs										
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a										
012100 Organic Chemistry										
13. START DATE			14. ESTIMATED COMPLETION DATE			15. FUNDING AGENCY		16. PERFORMANCE METHOD		
72 07			CONT			DA		C. In-House		
17. CONTRACT/GRANT					18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS		20. FUNDS (in thousands)	
A. DATES/EFFECTIVE: NA					B. PRECEDING		C. CURRENT		D. FUTURE	
B. NUMBER ^a					79		5.0		433	
C. TYPE:					FISCAL YEAR		CUMULATIVE		377	
D. KIND OF AWARD:					80		5.0		377	
E. AMOUNT:										
F. CUM. AMT.										
19. RESPONSIBLE DOD ORGANIZATION					20. PERFORMING ORGANIZATION					
NAME ^a Walter Reed Army Institute of Research					NAME ^a Walter Reed Army Institute of Research					
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RESPONSIBLE INDIVIDUAL					PRINCIPAL INVESTIGATOR (Furnish NAME if U.S. Academic Institution)					
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					SOCIAL SECURITY ACCOUNT NUMBER:					
21. GENERAL USE					ASSOCIATE INVESTIGATORS					
Foreign intelligence not considered					NAME: Canfield, C.J., COL MC					
					NAME:					
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Malaria; (U) Leishmaniasis; (U) Trypanosomiasis; (U) Schistosomiasis; (U) Antiparasitic Drugs; (U) Chemical Synthesis; (U) Antimalarials										
23. (U) The objective is to manage, integrate, and provide technical direction for both a contract and in-house program to obtain potentially active antiparasitic agents for military use through rational organic syntheses.										
24. (U) Necessary research areas are defined, proposed research evaluated, ongoing research guided, evaluated, and integrated with the other program elements. Technical advice is obtained through an Ad Hoc Study Group on Medicinal Chemistry. Information is exchanged by contractors through technical meetings.										
25. (U) Several outstandingly active antimalarial compounds have been obtained in the acridinedione and acridinedione imine series. Final testing in the Aotus monkey will determine at least one candidate of each class for pharmacological studies and IND preparation. Synthesis in these areas is being phased out. Outstanding activity was also obtained with new amodiaquin analogs; synthesis is being phased out and INDs are being prepared on two compounds. An outstanding 8-aminolepidine has been obtained; toxicity studies are being carried out and an IND is being prepared. Synthesis in this area is being phased out. Certain thiosemicarbazones continue to show modest antimalarial activity but, as a spin-off, outstanding wide spectrum, <u>in vitro</u> antibacterial activity. The synthesis of potential antileishmanial 8-aminolepidines has continued. The synthesis of potential antitrypanosomal and antischistosomal agents has been confined to nucleoside type compounds. During the year approximately 245 target antiparasitic compounds were submitted. Conversion of the old data processing system to a new fully integrated in-house system has continued. The inventory system has been completed and the chemistry system is progressing. For technical report see Walter Reed Army Institute of Research Annual Progress report, 1 Oct 78-30 Sep 79.										

Project 3M162770A803 DRUG DEVELOPMENT

Work Unit 084 Synthesis of Antiparasitic Drugs

Investigators:

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The Research Contract Chemical Synthesis Program

During this reporting period active contractual programs devoted to the synthesis of potential antiparasitic agents were divided as follows: malaria 9, leishmaniasis 2, trypanosomiasis 5 and schistosomiasis 2.

In the area of antimalarials work on the synthesis of potential hypoxanthine phosphoribosyltransferase inhibitors was phased out because of lack of activity, despite a very compelling biochemical rationale. A number of very good tissue schizontocidal 8-aminolepidines are now in hand and the intensity of synthetic work in this area has been diminished pending advanced testing results on the best compounds. Certain compounds in this class are extremely interesting in that they have both high tissue and blood schizontocidal activity.

Based upon the antiparasitic activity of a number of nucleosides, a modest synthesis program has been started to investigate several classes of tubercidin analogs. These include fluoro-substituted analogs and open chain analogs with polyhydroxy alkyl moieties in place of the ribosyl group. The same type of analogs of adenosine are also being investigated.

Work on the very active acridinediones and acridinedione imines, both in the 10-H and 10-OH series, has continued. The synthesis in these areas is being phased out because a number of excellent compounds have now been obtained and are now being evaluated against human malaria in the Aotus monkey. Synthesis of amodiaquin analogs has also been halted because three excellent compounds have been obtained; INDs are being prepared on two of these. The third is awaiting testing against human malaria in the aotus. Applications for patents will be made in all of these classes of compounds. Work has been started on a

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new class of active compounds, the indoloquinolines. This class shows much promise but phototoxicity may be a problem. Work has also been started on some bialamicol analogs to follow up on a prototype compound which showed good antimalarial activity without mefloquine cross resistance.

The synthesis of 6-substituted-2,4-diamino-5,6,7,8-tetrahydropyrimido(4,5-d)pyrimidines has been phased out. These compounds proved to be devoid of activity. The synthesis of potential antimalarials based upon derivatives of 2,3-dihydro-1,6-diazanaphthalene has proven to be chemically more difficult than expected. The basic ring system has now been synthesized and target compounds should be forthcoming soon. The chemical synthesis of the ring separated pteridine antifolate analogs has also proven difficult and no target compounds have as yet been synthesized.

In the area of antileishmanials, the synthesis of 8-aminoquinolines allied to WR 6026 [8-(6-diethylaminohexylamino)-6-methoxyepidine dihydrochloride] has continued to receive attention, albeit at decreasing level. Variations on the terminal function of the side-chain and nuclear modifications have been investigated but have failed to produce a compound superior to WR 6026 in anti-leishmanial effectiveness. Investigations have been initiated on 6-amino- and 7-aminoquinolines bearing the WR 6026 chain; no testing data are available at this time.

In addition to the aminoquinolines, phenazines allied to clofazimine are being investigated. It is still too early to evaluate the worth of this class of compounds.

Work on the synthesis of potential antitrypanosomal agents has continued. In addition to the ongoing work in the nucleoside area mentioned below, work was begun during the year on bis(sym-triazines) types and quaternary salts of diverse imidazo fused heterocyclic types. It is too early for an evaluation of these areas.

In addition to the nucleoside types being synthesized as potential antimalarials, analogs of nucleocidin and tubercidin are being synthesized as antiparasitics which would have potential as both antitrypanosomal and antischistosomal agents. The limited biological data available at this time on the nucleocidin analogs are quite encouraging with respect to antitrypanosomal activity.

Finally, a series of tetrahydrocarbazoles, related benzo-fused compounds and bis(hydrazides) are being synthesized and evaluated for their activity against T. cruzi. Insufficient data have accrued on this class to allow a judgment as to their worth.

Data Processing

Major programming efforts were undertaken in recent years to modernize the data processing systems. These systems update, store, and retrieve the chemical, biological, and inventory records necessary to the antiparasitic and antiradiation drug development projects of the Division of Experimental Therapeutics, WRAIR. Although created in support of Division needs, these records are of broad utility and are an asset of national import.

Capability has evolved from primitive, independently run IBM 1401 card/tape operations through partially integratable IBM 7090/94 systems toward fully integrated in-house CDC systems. The task of converting and integrating these systems is in progress and their current status is as follows.

1. Inventory

This system is fully operational and documented. Inventory is being updated every third day concomitant with the shipping cycle. Complete inventory reports on all samples are generated on tape and converted to microfilm periodically. Bottle (sample) number sequence reports are generated monthly while accession number sequence and source of sample sequence reports are generated quarterly.

2. Chemistry

Initial conversion of the chemical structure data base has been accomplished. Over 210,000 structures with their accession numbers are now indexed on this file. The first phase of verification (a comparison of the chemistry data base with the inventory data base) discovered over 50,000 unmatched accession numbers on the inventory file. The structures for these unmatched numbers must be (re) entered onto the chemistry file. This effort will continue into FY 1981. Further verification to account for all inventory samples can then be undertaken.

Integrated reports have been successfully generated from searches of the chemistry and inventory data bases and routine use is made of this limited capability.

Physical and operational modifications were made in the laboratory of the chemical shipping contractor in compliance with regulatory requirements.

Acquisition of Compounds

The following table summarizes the number of various classes of compounds received during FY 79.

	<u>Originals</u>	<u>Duplicates</u>	<u>Total</u>
Purchased	212	10	222
Gifts	112	22	134
Synthesized	491	195	686
Discreet	3215	361	3576
Prep Labs	31	40	71
Total	4061	628	4689

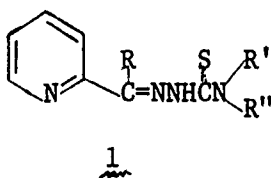
Eighteen sources submitted compounds under no-dollar agreements. The number of original samples increased by 9% over last FY, while the total number of samples received decreased slightly. The practice of sending out a collection team was re-instituted. Three companies were visited and 1181 samples were collected.

Organic Synthesis Section

An improved process for the purification of the antiradiation agent, WR 2721 (S-aminopropylaminoethylphosphorothioate), has been developed which gives a highly crystalline product, stable at room temperature. Work is currently in progress on devising a new purification procedure for WR 638 (sodium 2-aminoethylphosphorothioate) which will later be applied to the large quantity of the material on hand which was synthesized about 13 years ago. The compound is destined for use in the treatment of cystinosis.

Based on the findings that certain N^4, N^4 -disubstituted 2-acetylpyridine thiosemicarbazones possess "curative" activity at a dose of 40 mg/kg in mice infected with Plasmodium berghei, about 12 related derivatives were synthesized in which the

alkylidene side chain (R) of 1 is lengthened 1-4 carbon atoms beyond R=CH₃. Initial

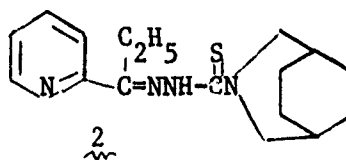


tests showed that such modifications reduced the toxicity of this group of thiosemicarbazones, however, it appears that anti-malarial efficacy also suffers as a consequence. In contrast, the antibacterial activity against some organisms increased markedly.

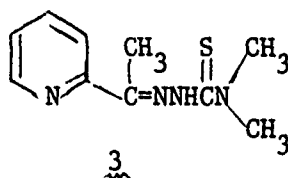
Several metal complexes of the most active 2-acetylpyridine thiosemicarbazones have been prepared. The transition metal ions include Fe³⁺, Ni²⁺, Co²⁺, Zn²⁺, and Mn²⁺. These chelates are undergoing final chemical analysis and will be submitted for biological evaluation shortly.

The preparation of a series of 1- and 3-acetylisoquinoline thiosemicarbazones is underway. Some related 2-acetylquinoline thiosemicarbazones were synthesized by Professor S. Massie and his students at the U.S. Naval Academy under our guidance. Early tests indicate that they are moderately active antimalarials and one compound, in particular, appears to be an excellent antibacterial agent.

Dr. Arthur Dobek of the Infectious Disease Service, Walter Reed Army Medical Center, has tested about 60 of the 2-acetylpyridine thiosemicarbazone against a greater number of strains than he had the previous year. Some of the new compounds which we have developed show vastly improved inhibitory activity in his screens. One of the more active is compound 2 which shows activity as follows (organism, MIC):



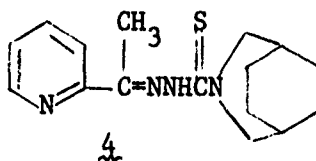
Staph. aureus, 0.125 µg/ml; Group D enterococcus, 0.25 µg/ml; Neisseria meningitidis, 0.016 µg/ml. The most inhibitory of the 2-acetylpyridine thiosemicarbazones against N. gonorrhoeae is compound 3 (MIC 0.004 µg/ml), which is the most toxic member of this group of thiosemicarbazones. We have now identified some 9 related compounds with considerably lower toxicity which have



MIC's in the range 0.004 to 0.125 µg/ml.

Additional 2-acetylpyridine thiosemicarbazones have been found by Dr. N. Morrison (Johns Hopkins) to be effective against Mycobacterium smegmatis. On the basis of his earlier work, three of our thiosemicarbazones are undergoing the six month mouse foot pad test against M. leprae. Results should be known in November. Because of their promising activity, further of our compounds will be undergoing the same test as soon as sufficient quantities of material are synthesized.

Inhibition by 2-acetylpyridine thiosemicarbazones of other mycobacteria (M. tuberculosis H₃₇R₆₁, M. avium, M. kansasii, M. simiae, M. marinum, M. intracellulare and M. habaña) is being studied by Dr. Frank Collins (Trudeau Institute). Four of the compounds screened look very promising against most of the mycobacteria. A mixture of 4, clofazimine, and rifampin

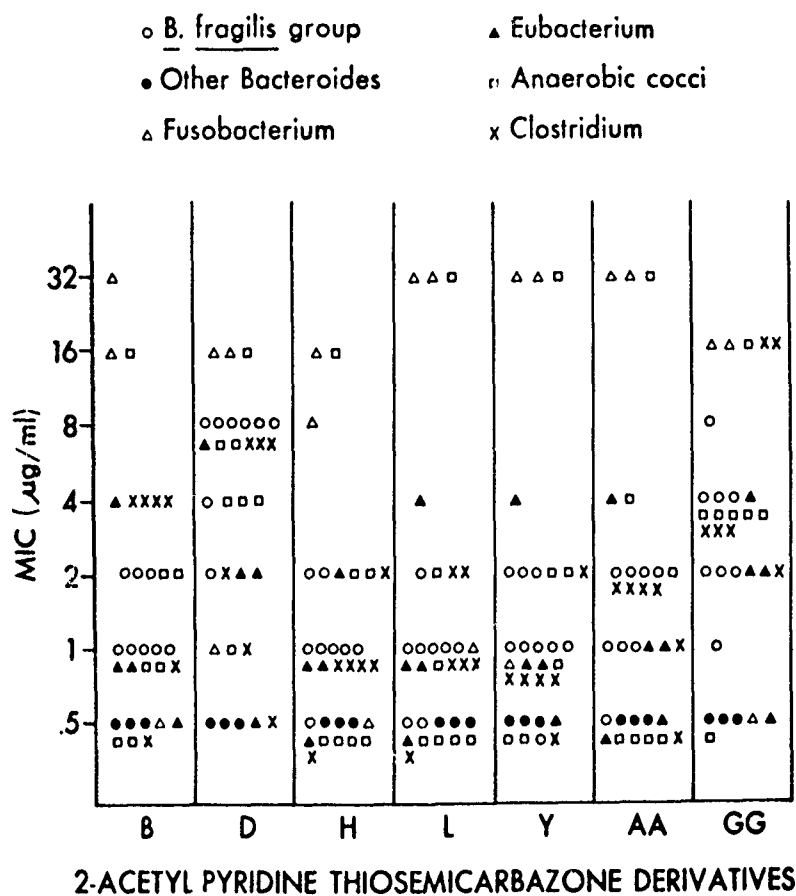


seems to have a potentiating inhibitory effect on the refractory atypical mycobacteria M. habaña and M. simiae in mice.

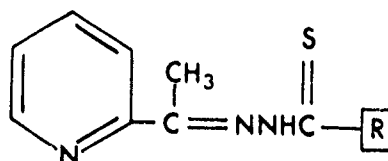
Anaerobic bacteria have been studied by Dr. J.E. Rosenblatt (Mayo Clinic) where 7 compounds have been found to have MIC's generally in the range of <0.5 to 1 µg/ml (cf. Table). This is approximately the same inhibitory concentrations at which known therapeutic agents work.

TABLE

Inhibition of Anaerobic Bacteria by Agents Developed at WRAIR



Structure Key to Previous Table



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A		R	
B		S	
D		V	
F		Y	
G		Z	
H		AA	
J		BB	
L		CC	
N		GG	
O		HH	

Over twenty 2-acetylpyridine thiosemicarbazones have been tested for antitrypanosomal activity in an in vitro screen. Activity was ascertained by the extent of inhibition of the uptake of radiolabeled thymidine and leucine by Trypanosoma rhodesiense. About half of the trial group that were studied possessed a high degree of activity comparable to the antitrypanosomal drug, ethidium bromide.

A small number of 2-acetylpyridine thiosemicarbazones have been studied by Dr. Charles Shipman, Jr. (University of Michigan) for their antiherpes simplex activity in an in vitro screen. Preliminary findings indicate that the compounds are sufficiently active to warrant the extension of the study to additional compounds. Preliminary studies have also been undertaken by Dr. E. De Clerq (Rega Institute, Belgium) who has reported that the few compounds examined do indeed possess antivaccinia and antiherpes action; however, the effective concentration appears to be too close to the cytotoxic concentrations. Additional compounds are undergoing screening at the Rega Institute where an investigation of their antifungal properties has also recently been initiated.

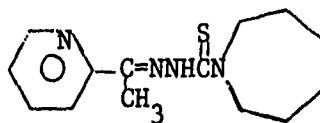
Using primaquine and its 4-methyl derivative as models, the 4-aminoacridinyl analogs 4-[(4-amino-1-methylbutyl)amino]-2-methoxyacridine and 4-[(4-amino-1-methylbutyl)amino]-2-methoxy-9-methylacridine were prepared and evaluated as potential tissue schizonticidal agents. These compounds were found to be less active than primaquine against Plasmodium cynomolgi in the rhesus monkey.

In an attempt to develop an assay for primaquine from biological fluids, primaquine from spiked solutions of water, urine or of blood was determined by gas chromatography (GC) and by high performance liquid chromatography (HPLC). The recovery by the GC method from spiked water was quantitative and a 74% recovery from spiked (10 µg/ml) urine was obtained. By HPLC, the recovery was quantitative from spiked water and 100%±20% from spiked urine or spiked (7 to 15 µg/ml) blood.

Using HPLC, a technique which permits a simpler procedure for isolation and clean-up than that needed for GC, primaquine shows a linear response between 1 to 102 µg/ml and a sensitivity level currently down to the 80 to 200 ng/ml range. Ongoing work is aimed at increased precision, accuracy and toward lower levels of detection and assay.

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The 2-acetylpyridine thiosemicarbazone, WR 231,010 (5), an



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antimalarial and antimicrobial agent, has been determined to be stable, for at least a week, in 50 vol % ethanol - 50 vol % 0.5 N HCl, a solvent used for some antibacterial tests. Under HPLC conditions suitable for primaquine, certain 2-acetylpyridine thiosemicarbazones can be analyzed readily.

Project 3M162770A803 DRUG DEVELOPMENT

Work Unit 084 Synthesis of Antiparasitic Drugs

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2. Collins, F.M., Klayman, D.L., and Morrison, N.E. Anti-mycobacterial Activity of 2-Acetylpyridine Thiosemicarbazones in vitro and in Chronically Infected Mice.

3. Bougault, A.-M., Gerdtz, A.M., Rosenblatt, J.E., and Klayman, D.L. In vitro inhibition of Anaerobes by 2-Acetylpyridine Thiosemicarbazones.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD DHA(LAR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DISSEM INSTR ^a	9. SPECIFIC DATA CONTRACTOR ACCESS ^a	10. LEVEL OF SUM ^a
78 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
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C. CONTRIBUTING	CARDS 114F						
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E. AMOUNT:							
F. CUM. AMT.							
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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(U) Toxicology; (U) Drug Development; (U) Antimalarials, (U) Biology;							
(U) Plasmodium; (U) Malaria; (U) Chemistry; (U) Pharmacodynamics; (U) Drug Metabolism							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code) ^a							
23. (U) The design, development, testing and exploitation of new antimalarials for military use against drug resistant strains.							
24. (U) Compounds active against malaria are identified by testing candidate drugs for activity in laboratory model systems of the disease. Information is used in formulation of new drug synthesis and in selection of candidate drugs for clinical trials. New laboratory test systems are developed.							
25. (U) Screening tests were done by in-house and contractor laboratories on approximately 8000 compounds in animal models or <u>in vitro</u> for suppressive, causal prophylactic or radical curative antimalarial activity. Approximately 500 compounds had activity. Approximately 100 of these were selected for advanced study, including 10 to be tested against human (<i>P. falciparum</i>) malaria in Aotus monkeys. The <u>in vitro</u> culture system with <i>P. falciparum</i> was used in evaluation of 40 candidate drugs and in study of metabolic requirements of the parasite during cultivation; 16 drugs were active <u>in vitro</u> and fatty acids were established as growth-supporting <u>in vitro</u> . For Technical Report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.							

^aAvailable to contractors upon originator's approval

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DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3M162770A803 DRUG DEVELOPMENT

Work Unit 086 Biological Evaluation of Antimalarial Drugs

Investigators:

Principal: LTC David E. Davidson, Jr., VC
Associate: CPT George E. Childs, MSC, Gerald J. McCormick,
Ph.D., Gloria P. Willet

1. Description.

Malaria is considered to be the leading health problem in the world and historically has consistently and seriously effected military operations in its areas of endemicity, which are global. In order to protect personnel and maintain effectiveness in field operations, new drugs are being sought which will be safer and more effective than those presently available. The requirement is urgent because of the emergence of strains of malaria which are resistant to usual drug treatments.

Candidate antimalarial compounds are screened and tested by the Department of Parasitology, WRAIR, and by contractor laboratories under the technical direction of the Department of Parasitology.

2. Progress.

a. Blood schizonticidal testing:

The discovery of new blood schizonticidal drugs for prophylaxis and treatment of drug-resistant falciparum malaria has been actively pursued in drug-testing systems. Primary screening at the rate of 120 compounds per week is conducted in the mouse/*Plasmodium berghei* blood schizonticidal test at the University of Miami. Approximately 6000 compounds were tested in the past year and, of these, approximately 500 compounds exhibited activity.

Approximately 200 of the active compounds were selected for further testing in vitro, in advanced rodent tests and in primate malaria models. Sixty compounds were tested for repository activity against *P. berghei* in mice; 15 compounds had schizonticidal activity at periods of a week or longer after administration. During this year, ten compounds with promise of clinical utility were selected for preclinical testing in the Aotus/*P. falciparum* model and testing was completed on four.

Schizonticidal testing in the rhesus monkey/P. cynomolgi model remains suspended due to non-availability of test animals.

b. Tissue schizonticidal testing:

Primary screening of candidate drugs for improved causal prophylactic and radical curative antimalarial treatments is performed at a rate of 45 compounds per week in the mouse model utilizing sporozoite-induced P. berghei yoelli malaria. Secondary mouse testing in the causal prophylactic model of Peters et al. is used to resolve ambiguities in activity which may arise in the primary screen by distinguishing between true causal prophylactic activity and the blood schizonticidal activity of persistent compounds. In this system in this past year, 21 compounds were examined. Seven had true causal activity against tissue schizonts, five had activity attributable to persistence, and nine were inactive. Agreement between the mouse systems and the rhesus monkey radical curative test has been approximately 80%.

The capacity for testing in the rhesus monkey radical curative test system has been reduced because the supply of animals has been severely curtailed. This system with sporozoite-induced P. cynomolgi malaria closely resembles P. vivax infections in man and allows the testing of true tissue schizonticidal activity. During this year, 21 compounds were tested in this system in a screening mode and of these, three exhibited sufficient activity to warrant more quantitative testing in this model.

c. In vitro antimalarial drug screen:

Efforts are continuing to refine the in vitro assay system in evaluation of antimalarial activities of drugs against chloroquine-sensitive (Uganda I) and chloroquine-resistant (Smith) strains of Plasmodium falciparum. Activity is measured as the inhibition of incorporation by the parasites of radioactivity from a labeled metabolic precursor (³H-hypoxanthine) of nucleic acid. A report describing the test system has been accepted for publication (1).

During the past year, approximately 40 compounds were tested for activity against the Uganda I and Smith strains; 16 were active. As anticipated, compounds of chemical classes which have activity in vivo as blood schizonticides against P. falciparum in the Aotus monkey proved to be active in vitro. However, compounds which had shown activity as tissue schizonticides against P. cynomolgi in rhesus monkeys were inactive in this

in vitro system.

A study was undertaken with standard antimalarial drugs in which the activity assayed by inhibition of uptake of radioactivity from ^3H -hypoxanthine was compared to the activity measured by reduction in parasitemia as determined morphologically. In both systems, parasites were cultured in vitro in microtiter plates for 36 hours in the presence of the drugs in serial dilutions. The concentrations of drugs corresponding to 50% effect on incorporation and parasitemia are presented in Table 1. There appears to be good correlation between the two assay methods.

d. In vitro malaria culture development:

Two strains of Plasmodium falciparum, chloroquine-resistant (Smith) and chloroquine-sensitive (Uganda), have been maintained in continuous cultures for up to five months. Samples are taken at intervals, glycerolyzed, and frozen. Reconstitution allows easy initiation of sub-cultures.

In order to investigate the growth requirements of the parasite and to develop culture media of defined constitution, cultivation studies are being done in which the plasma which is normally added to media is replaced by such constituents as fatty acids, dextran and fatty acid-free albumin.

Studies in which 1640 RPM medium contained added plasma at concentrations varying 0.6% to 40% showed that the best growth was achieved at the 5% concentration. In identical studies with added fatty acid-free albumin, no growth was observed at any concentration. A problem characteristic of media which contain plasma and are thus undefined was illustrated by an experiment in which growth was obtained in 1640 RPM medium formulated to be deficient in methionine, PABA, folic acid, isoleucine and threonine, but with added plasma. The absence of the deleted compounds should have had an adverse effect on growth but the plasma contained sufficient, but unknown, quantities of them. These results were obtained in both Smith and Uganda strains.

In studies in which 1640 RPM medium was employed with added fatty acid-free albumin rather than plasma, five different fatty acids were used in concentrations ranging from 10^{-5} to 10^{-2} mM. Of these, three appeared to support growth at 10^{-3} mM concentration. Combination of the three fatty acids resulted in good growth, as measured by the uptake of radioactivity of

³H-hypoxanthine by both Smith and Uganda strains in short term cultivations. When prolysin or dextran were substituted for fatty acid-free albumin in combinations with two fatty acids, there was no support of growth, except in a single experiment with dextran.

Subsequently, in long term cultivation of *P. falciparum*, a medium containing fatty acid-free albumin and a combination of fatty acids at 10^{-3} mM successfully supported growth for two weeks and achieved a 3200-fold increase in parasitemia (by serial increases).

e. Phototoxicity studies:

Phototoxic evaluations were performed on 13 compounds which had activity in antimalarial test systems. The compounds were of two structural classes: acridinophenones and 4-aminoquinolines.

In the test system, the compounds are administered to mice by IP and oral routes at several dose levels (the maximum tolerated dose (MTD), 1/2 MTD, and 1/4 MTD). The mice are exposed to light for a total radiation dose equivalent to 4.9×10^3 ergs/cm²/sec. Observation for toxicity commences immediately after exposure and is continued for 10 days. Vehicle and positive control groups are included.

Of seven 4-aminoquinolines tested (including five analogs of amodiaquine), only one compound, WR 242,449, was phototoxic. Extreme responses were seen at doses of 180 to 640 mg/kg by both IP and oral administration and the minimum dose at which response (erythema) was perceptible was 5 mg/kg.

Of six acridinophenones tested, four had phototoxic responses. WR 237,221 and WR 231,135 were positive by both IP and oral administration, WR 237,942 and WR 243,251 were positive by IP administration but negative by oral, and WR 243,246 and WR 226,626 were negative after both routes of administration.

f. Data processing:

Major efforts undertaken in recent years to modernize the process of biology file conversion and updating were brought entirely in-house upon the expiration in March 1979 of the contract with Advanced Computer Techniques, Inc., for biology data processing. Modernization efforts under this contract had had as their goals: (a) conversion from sequential access to

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random access capability with an increase in operational efficiency, (b) compression and standardization of data formats to reduce file size, (c) interface with chemical data system and inventory data system, and (d) modular design to facilitate the incorporation of additional kinds of biological data in the future.

However, the system was not fully operational as delivered by the contractor. Although each program had been tested with sample data by the contractor prior to delivery, the system as a whole had not been tested, nor had parallel processing with real data been performed prior to expiration of the contract.

By in-house efforts, conversion of the Rane biology file has been completed with biology data processing, file updating, and biology report generation now being accomplished as data are received. This file includes data from several historical in vivo antimalarial screens as well as the current in vivo antimalarial and antitrypanosomiasis screens. Conversion efforts are continuing on the non-Rane files which contain historical in vitro antimalarial screening data and in vivo data from resistant-strain malaria tests and, additionally, from antiradiation, leishmaniasis, and schistosomiasis screens.

Upon completion of the biology file conversions, programming for the integrated report generator will be extended to biology data. These efforts are expected to continue into FY 1981.

Table 1
Comparison of In Vitro Activity as Determined by
Inhibition of ³H-Hypoxanthine Incorporation and
Depression of Parasitemia

<u>P. falciparum</u> Strain	Drug	ED-50, ng/ml	
		³ H-Hypoxanthine Incorporation	Parasitemia
Smith	Chloroquine	75.2	105
	Mefloquine	5.68	7.86
	Quinine	80.4	60.7
Uganda	Chloroquine	1.26	1.79
	Mefloquine	3.05	0.83
	Quinine	36.4	n.d.*

*n.d.: not determined

Project 3M162770A803 DRUG DEVELOPMENT

Work Unit 086 Biological Evaluation of Antimalarial Drugs

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Publication, In Press:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD FORM 1498 (AR) 6 16	
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY ^a	6 WORK SECURITY ^a	7 REGRADING ^a	8A DISSEM INSTR ^a	8B SPECIFIC DATA - CONTRACTOR ACCESS	9 LEVEL OF SUM
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A. PRIMARY	62770A	3M162770A803	00	087			
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C. CONTRIBUTING	CARDS 114F						
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(U) Determination of Pharmacological Effects of Antimalarial Drugs							
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012600 Pharmacology 002600 Biology							
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C. TYPE				79		9.0	
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Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: CHUNG, Dr. H.			
				NAME: VON BREDOU, MAJ J.			
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(U) Antimalarial Drugs; (U) Preclinical Pharmacology; (U) Quantitation Methodology							
23. (U) The technical objectives are to develop and exploit animal models for the study of the pharmacodynamic and toxic effects of drugs intended for use as antimalarials in man. The intended purposes of these studies are to provide a basis for predicting human response and to fulfill requirements for submission of IND for clinical trials of new antimalarials for military personnel in malarious areas.							
24. (U) The approach is to study both the effects of antimalarial drugs on healthy animals and the fate of these drugs in healthy animals in order to predict the human tolerance to new drugs (Phase I). The handling of antimalarial drugs by diseased animals is being studied to determine the effects of malaria upon pharmacokinetics. This is in order to predict the tolerance of new antimalarial drugs in human efficacy studies (Phase II).							
25. (U) 78 10 - 79 09 Technical management continued for 13 contracts in pharmacology. Three new drugs were elevated to IND status in addition to the 14 IND's classified in the Active status. Intensive investigation into the factors affecting high pressure liquid chromatography results has led to the validation of that method for quantitating blood levels of WR 180,409. These levels were determined in hundreds of clinical samples. Pharmacokinetic studies on one antimalarial have been carried out in healthy human volunteers. An in vitro method for the determination of drug-induced methemoglobin formation has been developed. Further tissue distribution and pharmacokinetic studies were carried out in mice using radiolabeled antimalarials. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.							

^aAvailable to contractors upon originator's approval

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Project 3M162770A803 DRUG DEVELOPMENT

Work Unit 087 Determination of pharmacological effects of anti-malarial drugs

Investigators.

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1. Description.

Pharmacological investigations carried out by the department continue in two broad overlapping areas. One is the effect of the body or system on the drug, i.e., absorption, distribution, biotransformation and excretion. The second is the effect of the drug on the body or system, i.e., pharmacodynamics. In addition, continuation of the development and utilization of sensitive assay methods for several of the new antimalarial drugs was emphasized.

2. Preliminary refinement procedures for high pressure liquid chromatography of biological samples.

a. Background:

The quantitative and qualitative analysis of most pharmaceutically active compounds is relatively easy by means of modern analytical equipment if the compound is in its pure form and is dissolved in a pure solvent. If the chemical compound must be analyzed in a complex biological fluid, such as blood or urine, the entire procedure becomes exceedingly difficult. Before any form of analysis can be conducted, the compound must be isolated to an extent that it can be monitored in the presence of other materials within the same media. The ultimate analytical procedure must be sensitive and specific enough to detect the compound in the presence of other interfering materials. Therefore, at least one or more extraction and clean-up procedures will usually be required before most compounds can be subjected to a reliable analytical approach. Since the majority of the pharmaceutically active compounds can generally be divided into several chemically active groups, several broad spectrum extraction and clean-up procedures have been proposed. One very broad scheme has been proposed in the textbook of Clarke (1969). Once the

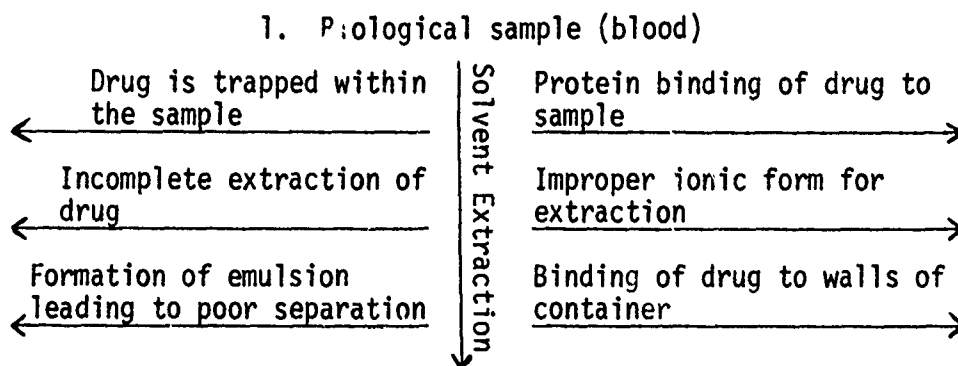
initial separation has divided the compound into one or more of several classes, the individual class of compounds may be analyzed directly or the class may be subdivided into more specific groups by various clean-up procedures. This systematic approach is very useful for most of the well-known pharmaceutical compounds.

The candidate antimalarials generally appear to possess nonspecific physical chemical properties resulting in very poorly defined isolation techniques. An extraction, isolation and quantitation technique has been developed by Grindel et al. (1977) for the antimalarial mefloquine (WR 142,490). Although this technique does extract at least 90% of the antimalarial and its internal standard, marked interference patterns often develop which may impose a significant limitation on the accuracy of this technique.

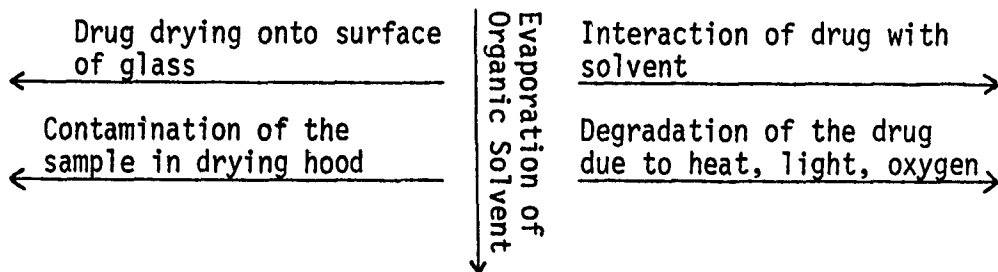
b. Possible sources of error:

Most of the work reported here has been carried out on the candidate antimalarial compound, WR 180,409·H₃PO₄ (DL-threo- α -piperidyl)-2-trifluoromethyl-6-(4-trifluoromethylphenyl)-4-pyridinemethanol phosphate) using as its internal standard the candidate antimalarial, WR 184,806·H₃PO₄ (DL-2,8-bis (trifluoromethyl)-4-[1-hydroxy-3-(N-t-butylamino)propyl]-quinoline phosphate). An extensive research effort on the possible complications which may occur in the extraction, isolation and quantitation of WR 180,409 and its internal standard WR 184,806 from spiked blood samples has been conducted. The anticipated complications are illustrated in the accompanying flow chart.

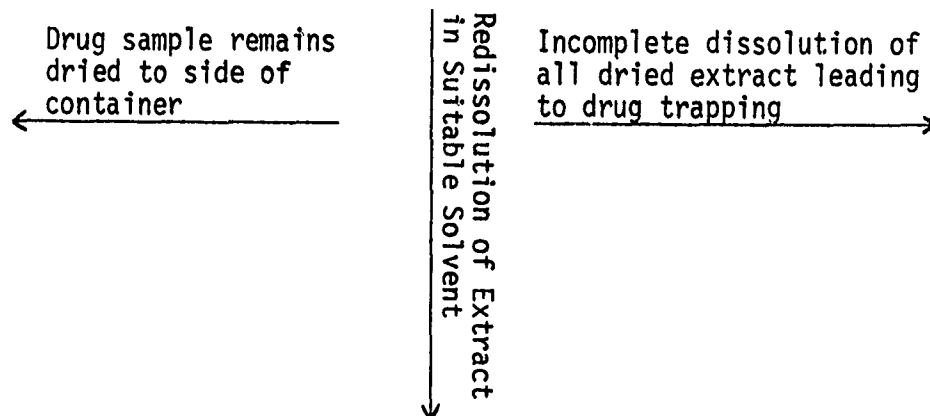
Flow Diagram Indicating Possible Sources of Error in the Extraction and Quantitation of an Antimalarial Agent from a Biological Sample



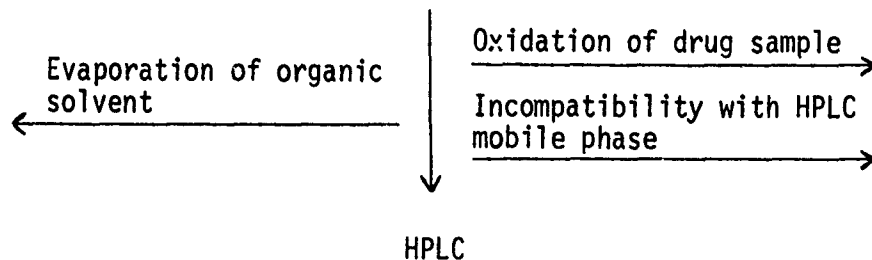
2. Dilute extract solution of the drug and biological extract in an organic solvent



3. Dry organic extract residue



4. Redissolved biological drug extract



c. Experimental:

The types of experiments listed below have been grouped in the manner indicated to aid the continuity of the basic principles of extraction, separation, detection and quantitation.

i. Treatment of the biological sample:

- a. equilibration with acid, base and buffer systems
- b. equilibration with 3M NaCl

ii. Extraction of the antimalarials from the biological sample:

- a. extraction with various solvents
- b. utilization of microcolumns to perform "clean up" procedures
- c. acid-base clean up procedures
- d. adsorption of antimalarial to solid medium
- e. detection of protein binding
- f. effect of heparin
- g. dye complexing

iii. Concentration of drug extract:

- a. antimalarial-solvent interaction
- b. refinement of concentrated biological extract

iv. Redissolution of the biological extract:

v. Sample injection:

vi. Chromatography:

- a. normal phase
- b. reverse phase
- c. multiple chromatographic procedures

vii. Detection:

viii. Quantitation:

Experiments were conducted to explore some of these potential complications. A detailed description of these experiments follows with the appropriate subsections of the list cross-referenced.

i. The biological sample is usually diluted with water, buffer or an aqueous solution of 3 M NaCl.

Equilibration with phosphate buffer affects the extent of recovery of the antimalarial and the internal standard as well as the interference pattern. The extraction efficiency of ethyl acetate with respect to the antimalarials WR 180,409 and WR 184,806 is illustrated in Table 1. A near neutral pH of 7.4 yields approximately equal concentrations of WR 180,409 and its internal standard WR 184,806. Thus this pH was chosen for the subsequent experiments.

The 3 M NaCl solution is often added to biological systems to disrupt and precipitate the proteins in order to liberate all of the available drug. No improvement in chromatographic peaks or total drug recovery was observed when the blood sample was equilibrated with 3 M NaCl.

ii.a. After the biological sample has been equilibrated with the phosphate buffer, the antimalarial is extracted with an organic solvent. The choice of organic solvent depends on the solubility of the antimalarial in that solvent. Solvents must be used in which the antimalarial is readily soluble but which are not miscible with water, thus eliminating the use of methanol or ethanol.

A primary property of the solvent is that it must extract the majority of the drug in a reproducible manner. In order to achieve at least 90% extraction of the antimalarial, multiple extractions are usually required. Most of the previous efforts in this laboratory have utilized three extractions with ethyl acetate. This has resulted in the extraction of the antimalarial and the chosen internal standard, but has also led to the extraction of massive amounts of unknown biological components which interfere in the final chromatographic analysis.

An attempt was made to use other organic solvents which might be substituted for ethyl acetate as an extractant. Tables 2, 3 and 4 illustrate the effectiveness of ethyl acetate and other organic solvents to extract WR 180,409 and its internal standard WR 184,806 from blood equilibrated with either phosphate buffer or 3 M sodium chloride and the apparent usefulness of the resulting HPLC chromatograms.

Since most organic solvents are at least minimally soluble in water, some of the aqueous phase of the biological sample will be transferred into the organic solvent layer during the extraction. As the organic solvent is concentrated by drying,

interfering substances dissolved in the aqueous phase may be trapped in the organic extract which will ultimately add to the final chromatographic interference pattern. In attempts to limit this possibility, the organic solvent used to extract the antimalarial from the biological sample can be "washed" with a clean aqueous phase. The ethyl acetate extracts containing WR 180,409 and WR 184,806 from a biological sample were extracted with two 10 ml portions of pH 7.4 phosphate buffer. The resulting "washed" ethyl acetate extracts were concentrated by evaporation, redissolved in an appropriate mobile phase and chromatographed by means of HPLC in the usual manner. The interference patterns near the antimalarial chromatographic peaks were approximately the same as before and essentially no improvement in peak quantitation was observed. Although no improvement was observed in these experiments, it may be possible to use a combination of buffer mixed with a very polar organic solvent which may remove some interfering substances without loss of a significant amount of drug.

ii.b. Interference resulting from unknown biological components within the organic extract is a common problem which has been approached commercially through the production of miniature extraction columns designed to eliminate some of the major interference patterns.

A commercially available extraction column, SEP-PAK (Waters Associates, Inc., Milford, Mass.) is available in two different packing materials.

Silica SEP-PAK

A silica SEP-PAK was prewashed with a solution of ethyl acetate saturated with pH 7.4 phosphate buffer.

The ethyl acetate extract of the biological sample was passed through the SEP-PAK three times in order to allow sufficient contact with the adsorbent column contents. After the ethyl acetate extract containing the antimalarials WR 180,409 and WR 184,806 was passed through the SEP-PAK for the third time, HPLC analysis of the eluate indicated that both of the antimalarials had fully adsorbed onto the column. The silica SEP-PAK containing the antimalarials was then flushed with additional ethyl acetate, followed by hexane, and ultimately with ethylene dichloride. HPLC analysis of the washes indicated that neither WR 180,409 nor WR 184,806 were removed from the SEP-PAK column by these clean up procedures. These procedures did remove some of the major interference materials in the final chromatographic

analysis. The antimalarials were then eluted from the SEP-PAK column with three separate aliquots of methanol. The first 10 ml aliquot of methanol contained the majority of WR 180,409, as is indicated in Table 5, but unfortunately, it also contained a significant degree of interference. The second 10 ml aliquot demonstrated less interference but contained only a small quantity of WR 184,806 (<20%). The third 10 ml aliquot eluted a small interference peak as well as an insignificant amount of WR 184,806 (<10%).

The SEP-PAK was further eluted with warm (50°C) methanol, resulting in no further recovery of the antimalarial but a significant amount of interference. Additional extraction with hot (60°C) 50% MeOH/50% H₂O as well as final extraction with a solution of 0.1 N HCl did not result in the recovery of additional WR 180,409 or WR 184,806. Therefore, even though the silica SEP-PAK is capable of extracting the antimalarials from the ethyl acetate extract, and a significant amount of the major interference can be eliminated, the final chromatogram could not be improved to a significant extent.

The equilibrium of extraction and retention of the antimalarials within the silica SEP-PAK may be altered by the polarity of the solvent used to place the extract onto the column. In a separate experiment the ethyl acetate extract was concentrated by drying and the solids redissolved in dimethyl formamide (DMF), a very polar organic solvent. The DMF solution of drug extract was washed through the silica SEP-PAK column three times in a manner similar to the previous ethyl acetate extract experiment. Upon chromatographic analysis (Table 5) it was determined that the majority of the antimalarials remained in the polar organic solvent and were not extracted by the SEP-PAK. Similar "clean up" steps were applied to this silica SEP-PAK. The methanol elution of the micro column demonstrated an insignificant degree of recovery of the antimalarial.

Since the antimalarials were not adsorbed from the polar solvent, DMF, a less polar solvent, ethylene dichloride, was tried. The ethylene dichloride containing the extracted antimalarials was flushed through the silica SEP-PAK as before. Chromatographic analysis of the remaining ethylene dichloride indicated that all of the antimalarials had been adsorbed by the column. The corresponding chromatograms demonstrated only a very small interference pattern, suggesting that the silica column had extracted not only the antimalarials but the majority of the interference as well. The SEP-PAK column was then washed with additional ethylene dichloride, without loss of the

antimalarial. The SEP-PAKs were eluted with three separate 10 ml aliquots of methanol which resulted in a recovery of approximately 60% WR 180,409 and 90% WR 184,806. Additional elution with warm methanol and methanol-water yielded additional recovery of WR 184,806 but very little, if any, WR 180,409. Thus although essentially all of the WR 184,806 can be removed, only 65% of the WR 180,409 can be eluted from the silica SEP-PAK. Unfortunately the chromatograms corresponding to the various elution techniques were not significantly more independent of interference patterns.

C18 SEP-PAK

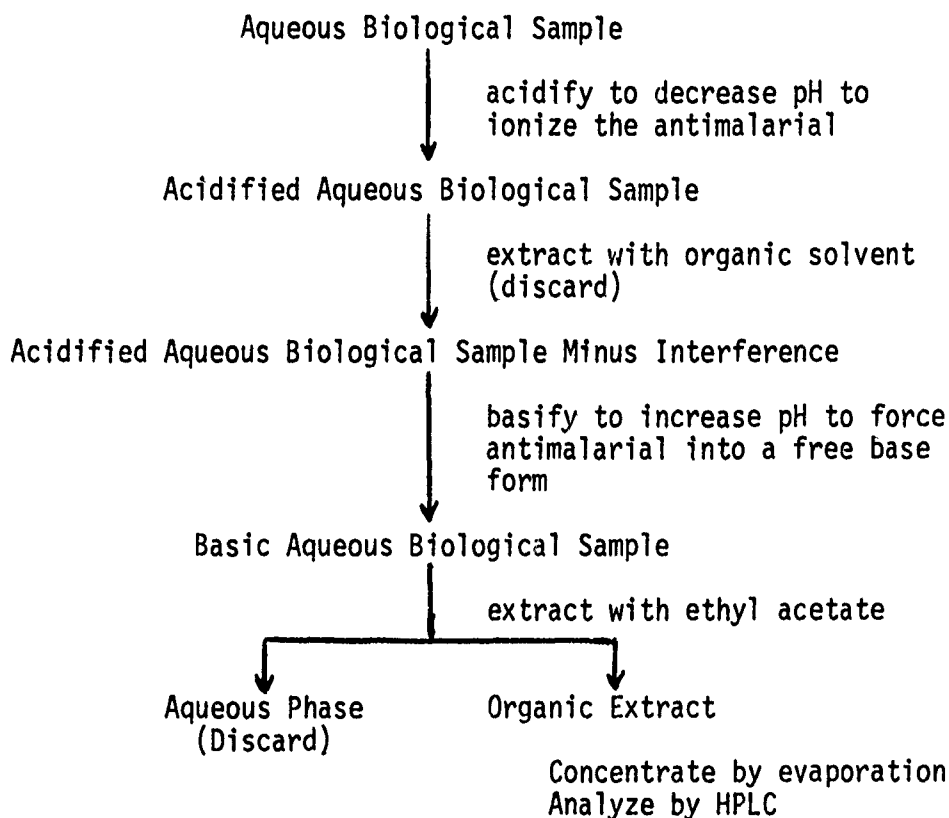
In two separate experiments the concentrated ethyl acetate extract was dissolved in DMF or an equal part mixture of DMF and water and placed on a C18 SEP-PAK. The C18 SEP-PAK system was then washed with additional DMF resulting in the loss of some antimalarial. Elution of the remaining antimalarial from the column was attempted with methanol. Only limited quantities of the remaining antimalarial were removed with methanol and the resulting chromatograms included a significant interference pattern.

ii.c. In the already established procedure for extracting the antimalarials, 5 ml of blood are equilibrated with 5 ml of pH 7.4 phosphate buffer and the resulting suspension is extracted three times with 10 ml aliquots of ethyl acetate. The ethyl acetate fractions are collected, combined and concentrated by evaporation. The concentrated extract is redissolved in an appropriate mobile phase and analyzed by HPLC. The analysis by means of HPLC is complicated significantly by the presence of interference patterns due to unknown materials extracted from the blood. Under ideal conditions it should be possible to "wash" some of these interference impurities out of the ethyl acetate extract. Since ethyl acetate is miscible with many other organic substances, it is very difficult to refine the ethyl acetate extract. An initial attempt was made to wash the extract with additional pH 7.4 phosphate buffer as reported in subsection i.a. Although no improvement was observed by this technique, no loss of the antimalarial was observed.

Most of our current antimalarials are weak amines which should become ionized in acid solutions. The ionized form must be sufficiently water soluble and therefore must remain in the aqueous phase during partition with an organic solvent. If the antimalarial can be forced to remain in the aqueous phase by means of ionization then it may be possible to extract the biological sample with an organic solvent capable of

removing the interfering substances. When the interference has been removed, the pH of the remaining aqueous phase can be increased until the antimalarial returns to its water insoluble unionized form. Under these conditions the amine can be extracted from the aqueous phase in the usual manner and analyzed by HPLC as before. The final chromatograms should contain considerably less interference since the usual interfering substances should have been removed by pre-extraction with organic solvents.

The general concept is illustrated in the following flow sheet:



The most ideal acid-base "clean-up" technique would utilize ethyl acetate to remove all of the normal interference while the antimalarial is in its ionized water soluble form. As the pH is increased to convert the antimalarial to its water insoluble free base form, ethyl acetate would again be used to extract the antimalarial from the remainder of the biological sample. Several variations of pre-extraction with organic solvent were attempted; the results are described in Table 6.

The ionized form of the antimalarial is apparently somewhat soluble in the organic layer. This was already suggested by the lack of retention of the antimalarial at low pH in the initial recovery experiments described in subsection i.

A small consistent loss of both antimalarials can be tolerated and accounted for if there were a dramatic improvement in the HPLC chromatogram. Only minimal improvement, if any, was observed in these experiments, however.

ii.d. A recent article (Soldin et al., 1979) has described a method for the adsorption of ritilinic acid onto charcoal, separation of the charcoal-ritilinic acid from the interfering components, liberation of the ritilinic acid from the charcoal and ultimately quantitation of the ritilinic acid by HPLC analysis.

A similar approach was attempted by adding the activated charcoal "Norite" to the ethyl acetate extract of the antimalarial. After prolonged mixing the charcoal was separated from the ethyl acetate by centrifugation and then washed with additional ethyl acetate. The Norite was ultimately extracted with methanol to recover the antimalarial for analysis. In one experimental trial all of the antimalarial had been removed from the ethyl acetate extract; however, it was not possible to remove the antimalarial from the Norite with several methanol extractions.

The experiment was repeated using silica instead of Norite charcoal. Unfortunately the silica removed only 75% of the antimalarial from the ethyl acetate, and extraction of the silica with methanol recovered only 25% of the total antimalarial. Total recovery of the antimalarial was increased to 40% when dilute hydrochloric acid was added to the methanol.

Although these initial results are not encouraging, many other forms of charcoal, silica and adsorbents are available which may be able to selectively remove the antimalarial from the biological sample and may be attempted in future experiments.

ii.e. Most of the candidate antimalarials have some degree of protein binding, which may prevent complete extraction of the antimalarial leading to very erratic results. Protein binding can often be disrupted by equilibration of the biological sample with 3 M NaCl as mentioned previously, or by freezing (Newell et al., 1978). Since all of the validation experiments are

conducted utilizing outdated bank blood spiked with methanol solutions of the candidate antimalarial, it may be that the compounds do not bind with the protein in its normal in vivo manner and binding values may be misleading in the usual in vitro validation study. The analysis of a difference in in vivo and in vitro binding of these antimalarials may only become apparent with radiolabeled experiments in animals.

Several experiments were conducted to determine if part of the massive interference peak in the ethyl acetate extract was due to protein. If proteins are present in the organic extract they can usually be precipitated with acetonitrile, acetone or trichloroacetic acid. No precipitate was noted when 3 ml of acetone or 3 ml of acetonitrile was added to a 10 ml ethyl acetate extract. Three ml of a 20% trichloroacetic acid added to a 10 ml ethyl acetate extract did not induce a precipitate to form while all three of these solutions produced a white precipitate in the unextracted plasma sample.

If the protein components are broken down by the extraction procedure to several amino acids, it is possible that these may be extracted and become a significant part of the chromatographic interference pattern. Since the majority of the amino acids do not possess UV absorption in the normal analytical range, no contribution to the interference pattern would be expected. However, tryptophan possesses significant UV absorption in the analytical range. Dilute methanol solutions of tryptophan were injected into the HPLC, resulting in a lack of significant retention on the micro bondapak CN column. Therefore its UV absorption pattern would not be expected to interfere with the detection of the antimalarial peaks.

ii.f. Heparin is a common anticoagulant used in many human protocols. Extraction of the heparin with the ethyl acetate could also contribute to the interference pattern, which may be easy to eliminate by changing to another anticoagulant.

Five ml of dog blood were extracted with ethyl acetate immediately after collection; 0.2 ml heparin was added to 5 ml of blood collected from the same dog and was extracted in the same manner as the first blood sample. The HPLC interference pattern of both blood samples were identical. Therefore, if heparin does induce an interference pattern it is not expected to interfere with the chromatography of the current antimalarials.

ii.g. Another technique which may aid in the extraction of the antimalarial from the ethyl acetate extract utilizes the

amine functional group. The amine function of many compounds can complex with other reagents leading to the formation of a colored product which is readily quantitated by UV spectrophotometry. Both WR 180,409 and WR 184,806 complex with the acid dye bromthymol blue which can then be extracted from the aqueous media with a very nonpolar solvent such as cyclohexane. The cyclohexane can be concentrated by evaporation, simplifying the HPLC process. A similar response was observed with congo red and thymolsulfonphthalein.

In a basic experiment designed to demonstrate the ability of a dye complex to form, bromthymol blue and congo red were allowed to interact with WR 180,409 and WR 184,806 in the presence of a dried ethyl acetate extract which had been redissolved in a 50% DMF/50% water solution. After mixing for one hr, the resulting solution was extracted with cyclohexane. Either the acid dye complex did not form under these conditions or the complex could not be extracted with cyclohexane.

iii.a. In the current procedures, the extraction step involves approximately 30 ml of organic solvent. In order to concentrate the organic solution of the antimalarial and its internal standard, the organic solvent is evaporated; this causes the drug, the internal standard and interference material to be deposited along the glass sides of the vessel. The entire dried drug extract is then redissolved in 0.5 to 1 ml of the organic solvent mixture utilized as a part of the mobile phase in the HPLC system. Under these conditions the drug and the internal standard, as well as the interference materials, have been concentrated at least 30-fold. Since the majority of the extract consists of interference materials that have been solubilized in an organic solvent of very specific polarity, it may be difficult to redissolve all of the interfering materials in a solvent mixture of different polarities as may be required by the HPLC system. In order to be certain that none of the drug or its internal standard are trapped among the interfering substances, it is imperative that the entire extract be completely solubilized by whatever solvent is chosen to redissolve the extract. The small quantity of organic solvent chosen to redissolve the extract must come in contact with all of the glass surface of the inside of the tube. This may be very difficult if the quantity of organic solvents required to dissolve the extract is extremely small. Several washings of the inside of the glass vessel with the organic solvent and then reconcentration by further evaporation may be required.

Another limiting factor in the redissolution of the extract and the antimalarial is the propensity of some of the antimalarials to stick to the glass surface. If the adhesion of the antimalarial to the glass surface is relatively strong, it may be very difficult for the limited quantity of organic solvent to redissolve the solids. Concentrating the antimalarials and the extraneous materials in the extract by evaporation may promote solvent interaction with the antimalarials and other materials within the extract. An apparent interaction between the antimalarial and the organic solvent was noticed in several experiments and was directly dependent on the amount of time required to evaporate the organic solvent. The longer the organic solvent was allowed to remain in contact with the antimalarial during this evaporation phase, the greater was the apparent loss in the final chromatographic analysis of the antimalarial and its internal standard. This is shown in Table 7. This phenomenon was much more apparent if the antimalarial and the internal standard were allowed to remain in contact with only the organic solvent and no interfering materials from the normal blood samples were present. This suggests that the interfering substances which are also extracted by the organic solvent may actually trap the antimalarial and its internal standard and thereby keep it from interacting with the organic solvent. Thus, the interfering materials may actually serve a beneficial purpose. If the interfering materials complex with the antimalarial and the internal standard, this may allow the antimalarial and its internal standard to be placed on the HPLC column for analysis. Unfortunately, the interfering materials affect the final chromatography of the antimalarial and its internal standard and make the final quantitation very difficult. The possible adhesion of the antimalarial to the glass and the possible interaction of the antimalarial with the extracting organic solvents or the organic solvents utilized to place the sample on the HPLC system may severely limit the ability to develop a refined clean-up system. The improved quantitation which should be achieved by a refined extraction technique may be lost due to the interaction of the more pure antimalarial with the organic solvents or by the adhesion to the glass surface. In several experiments, every effort was made to minimize the contact between the antimalarial and the sides of the glass vessel wall, thereby limiting the effect to interaction between the antimalarial and the organic solvent. Under these conditions, only a very limited amount of drug could be recovered. In other experiments involving the same amount of organic solvent but maximum contact with the sides of the glass vessel wall, more drug was ultimately recovered. Thus, it appears as though

adhesion to the sides of the glass vessel wall may be beneficial; i.e., if the antimalarial has had an opportunity to adhere to the glass walls, it will not interact with the organic solvent and may ultimately be recovered by dissolution into the mobile phase. The interaction which is observed is between the organic compound, ethyl acetate, and the antimalarials. Ethyl acetate treated in an identical manner has no interference spectra whatsoever.

Table 7 also demonstrates the use of other organic solvents as a possible substitute for ethyl acetate. These solvents were allowed to remain in contact with the antimalarial for 72 hr. The organic solvent was then evaporated to dryness and the extract was redissolved in a small quantity of the appropriate mobile phase. All of the organic solvents utilized appear to have some interaction with the antimalarial, except possibly hexane. In spite of a lack of interaction between hexane and the antimalarials, the ultimate recovery of the antimalarials was poor. The organic solvent xylene, or a mixture of xylene and methanol, allowed the greatest recovery of the antimalarials; however, it also induced a marked interference pattern. Therefore, the amount of chromatographic interference does not appear to be directly related to the amount of interaction between the organic solvent and the antimalarials.

iii.b. Since the extraction of WR 180,409 and WR 184,806 with other organic solvents was not as successful as extraction with ethyl acetate, attempts were made to simply "clean up" the already dried, concentrated ethyl acetate extracts. The dried ethyl acetate extract was dissolved in 5 ml of the very polar solvent, dimethylformamide (DMF) and the resulting solution was extracted for one hr with 10 ml of the very non-polar solvent, hexane. The hexane was separated from the DMF solution of the extract. Both the DMF solution and the hexane were concentrated by evaporation and redissolved in the appropriate mobile phase for HPLC analysis. The hexane does remove some of the interference from the beginning of the chromatogram as well as a small, but tolerable, amount of the antimalarials. Unfortunately, the hexane removes very little of the interference near the critical area of the chromatographic peaks. Therefore, the chromatograms of the DMF solution remaining after the hexane extraction do not demonstrate a significant degree of improvement.

iv. In order to avoid solubility complications, a concentrated biological extract of the antimalarials should be dissolved in an organic solvent which is the same as or very

similar to the mobile phase being used in the HPLC system. In normal phase HPLC, this is usually a mixture of ethylene dichloride and hexane, which readily dissolves not only the antimalarials but all of the interfering substances as well. If the reverse phase is to be used, a solvent compatible with the highly polar mobile phase must be selected which will also dissolve all of the organic extract. Solvents such as methanol which are highly polar and compatible with the reverse phase mobile phase have great difficulty in dissolving the highly lipophilic organic extract. Although the antimalarials are very soluble in methanol, they may be trapped by the insoluble material within the extract unless the extract is entirely dissolved.

Difficulty in redissolution of the biological extract may often be a function of the necessarily very limited amount of organic solvent used. The small amount of solvent limits the solubility of the extract, and complete contact with all of the antimalarial remaining on the sides of the glass tube is almost impossible. In spite of constant rotation and ultrasonic agitation it is possible that some of the antimalarial may remain attached to the sides of the glass vessel.

Prevention of adhesion to the sides of the glass tube has been attempted through the silization of the glass tubes without any significant effect.

Improved solubilization and chromatography has been achieved by increasing the total volume of the solution used to dissolve the sample and by increasing the polarity of the injected sample with respect to the polarity of the mobile phase of the column. The solid residue can readily be dissolved in 1.0 ml absolute ethanol at room temperature. When 0.5 ml acetonitrile is added to this ethanol solution of the biological extract, a fine white precipitate is formed. The degree of precipitation is increased significantly when this sample of 5 ml is rapidly frozen by suspending the tip of the round bottomed test tube in a mixture of dry ice and acetone. The mixture is then centrifuged at -25°C for 15 min, resulting in a solution containing copious amounts of a fine white precipitate. A small quantity (50 μl) of the solution is injected into the normal phase HPLC system. The resulting chromatogram contains less interference and somewhat better separation of the chromatographic peaks. This technique may ultimately be useful in the development of a final method.

v. After the evaporated extract has been redissolved in an organic solvent compatible with the mobile phase, an aliquot

of it must be injected into the HPLC system. (In the HPLC apparatus used in these experiments, it was possible to inject any quantity of the sample up to 3 ml.) Usual injection volume is between 5 μ l and 50 μ l; for good results, the accuracy of the injection of the sample is critical. In order to avoid an error in the injection volume or possibly a loss of some of the sample volume within the injector, an internal standard is added to the original biological sample. For our purposes, the internal standard is another antimalarial with properties similar to the compound to be analyzed. In this manner the internal standard is treated exactly the same as the compound to be analyzed. In the final analysis, the unknown compound is compared directly to the internal standard. In this manner, the internal standard should be able to compensate for inaccuracies and thereby correct for any inaccuracies in the injection of a fraction of the biological extract.

vi. The primary function of the chromatographic column of the HPLC system is to separate the antimalarials from other interfering substances which are present in the extract of the biological samples. The current candidate antimalarial agents may respond to several different types of column packings. A column filled with a relatively polar packing will have some attraction for the semipolar antimalarial without exhibiting a significant degree of attraction for the nonpolar lipid components. The antimalarial agents can ultimately be eluted with a semipolar mobile phase. Under ideal conditions it may be possible to select a mobile phase which is composed of several organic solvents so that the final polarity enables one to remove the interference components first and then remove the antimalarial components at a later interval with the same mobile phase. This is often designated as "normal phase chromatography" and can be accomplished through the use of a 5 component solvent mixture (Stampfli et al., 1979) and a Waters Associates microbonda-PAK CN column.

The "reverse phase" approach can be achieved by binding the antimalarial agents to a nonpolar column followed by differential elution of the antimalarials and the interfering components with a polar mobile phase. The reverse phase is somewhat less expensive to operate and has generally been accepted as being capable of more consistent results. Unfortunately, solubility differences of the biological extract and the required polar mobile phase have made this system difficult to apply to the antimalarials.

In order to eliminate the major interference components a dual column chromatographic procedure was proposed by

Sparacino (1977). In this procedure an initial separation of the antimalarial and the major interference components are made on the first column. The interference components are channeled to "waste" in the normal manner while the antimalarial fraction is shunted to a second column by means of a switching valve. A final, more precise separation of the antimalarials can then be made on the second column. This technique was not successful in the analysis of WR 30,090 but may be considered for the analysis of other candidate antimalarials.

A variation of the Sparacino technique is to perform an initial separation of the antimalarial fraction from the major interference components. The antimalarial fraction from the HPLC is collected, concentrated by evaporation and then rechromatographed on a separate column utilizing a mobile phase specifically suited for each chromatographic step. This more complete separation should eliminate the interference components and allow more accurate quantitation of the antimalarials.

Our initial efforts to quantitate the antimalarials by this two step technique have not been successful. Unfortunately an irregular source of loss of the antimalarial WR 180,409 was encountered. Repetition of these experiments with radiolabeled WR 180,409 will be required to determine the source of error.

vii. After the antimalarials have been separated by the chromatography column they must be qualitatively and quantitatively identified. The ideal detector would respond only to the drugs of choice and would not respond to any of the interference components. Unfortunately, very few specific detectors are available and the specificity of the detector required is entirely dependent on the compound to be analyzed. Fortunately most of the antimalarials have a high inherent absorptivity of ultraviolet light and can be monitored at the standard detector wavelengths of 280 or 254 nm. Most of the solvents used in normal phase chromatography are transparent at these wavelengths but absorb significantly at lower wavelengths. Although the antimalarials absorb an adequate amount of ultraviolet light at the aforementioned wavelengths it should be possible to achieve even greater sensitivity at wavelengths as short as 220 nm. Only reverse phase chromatography can be used in this area of the ultraviolet spectrum since only a few highly polar solvent systems are transparent. Unfortunately the chromatographic interference components also absorb ultraviolet light more intensely at 220 nm. This may invalidate any apparent advantage achieved by utilizing more specific absorbance wavelengths.

viii. The response of the ultraviolet detector to the drug peak eluted from the chromatographic column is monitored by a chart recorder. The area of the recorded peak is directly proportional to the concentration of the antimalarial within the sample. Within limits of ultraviolet spectrophotometry this linear relationship is consistent and can be used to determine the concentration of the chromatographed antimalarials.

Under ideal conditions the area of the chromatographic peaks of the biological extract can be compared directly with the area of the peaks produced by similarly chromatographed methanol standards. Unfortunately this comparative relationship can only be assumed for nearly perfect chromatography. Any deviation induced by chromatographic interference may invalidate the quantitative relationships and lead to irregular results.

Determination of the area of the chromatographic peaks has always been attempted by simple triangulation. This system leads to very consistent results whenever the chromatographic peaks are near optimum; however, any deviation from symmetrical peaks invalidates the technique. The application of modern integration techniques will be attempted as soon as feasible.

The development of procedures leading to more refined samples for injection into the high pressure liquid chromatographic procedure which will ultimately result in a more accurate analytical technique is the objective of most analytical laboratories and will be a continuing effort. Although many systems have been examined in these experiments, none have really been successful or have demonstrated marked improvement. Currently it is the combination of every possible technique which allows this system to function and is therefore dependent on the nearly optimal operation of each individual step. Difficulty in any of these steps may lead to a significant amount of error in the final analytical procedure.

3. The analysis of WR 180,409 in human blood samples by means of high pressure liquid chromatography.

a. Background:

Blood and tissue concentrations of the new candidate antimalarial, WR 180,409, have been determined in these laboratories by means of high pressure liquid chromatography, as well as with radiolabeled forms of WR 180,409. The use of radiolabeled isotopes is very accurate and simple; however, this

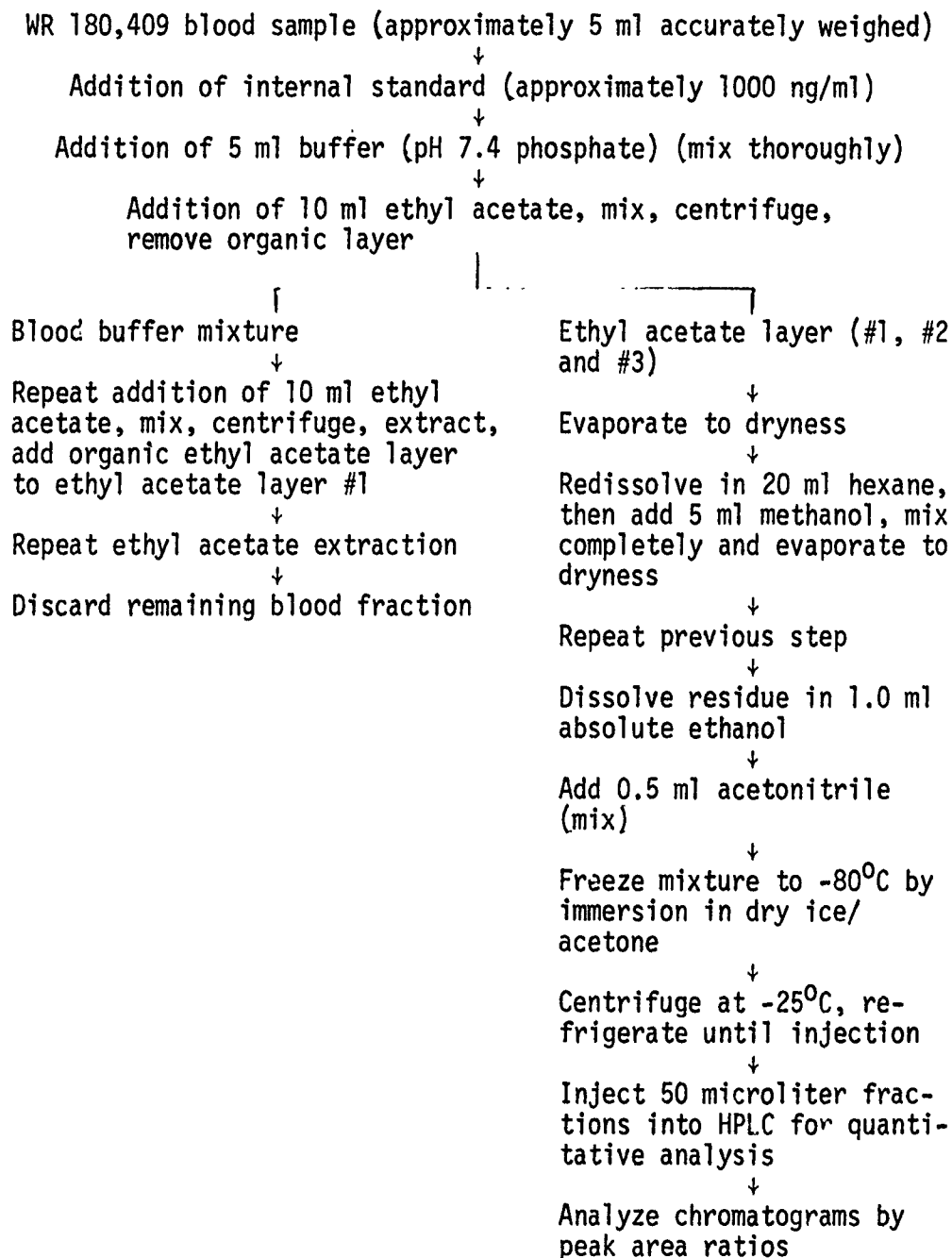
technique cannot be used to determine blood levels of this candidate antimalarial in the human volunteer.

The previous approach to high pressure liquid chromatography had led to irregularities due primarily to the interfering materials in the organic extract. Many experimental attempts have been made to improve the extraction of the antimalarial and to develop techniques which may refine the organic extract for more suitable use in high pressure liquid chromatography. A number of these attempts have been reported in section 2. The improved extraction techniques as well as the refinement procedures have been combined into a technique for the analysis of WR 180,409. Although this is not the ultimate technique, and further improvements in sensitivity, precision and accuracy are anticipated, it does serve as a working concept for basic clinical application.

b. Experimental:

The basic principle of this technique is to add the internal standard (WR 184,806) to the blood sample in a concentration of approximately 1000 ng/ml. The entire blood sample of approximately 5 ml is equilibrated with 5 ml of a phosphate buffer at pH 7.4; the mixture is then extracted three times with 10 ml ethyl acetate each. The three portions of ethyl acetate are combined and concentrated to dryness by evaporation at slightly elevated temperatures with a gentle flow of ambient air. The extract is redissolved in 20 ml hexane and then equilibrated with 5 ml of methanol. This mixture is again concentrated by evaporation to dryness; the procedure is then repeated once and the dried residue is redissolved in 1 ml of absolute ethanol. Upon complete dissolution of the residue in the ethanol, 0.5 ml of acetonitrile is added, whereupon a slight white precipitate forms. The sample is frozen at -80°C by immersion in dry ice and acetone. The white flocculent precipitate is then forced to settle by centrifugation of the mixture at -25°C, resulting in a clear yellow solution with a white more tightly packed precipitate at the bottom of the tube. A 50 µl portion of the clear solution is then injected into the HPLC system. This combination of acetonitrile and ethanol is compatible with either the normal or the reverse phase HPLC system. To date, all of the samples have been analyzed by means of the normal phase system on a Waters Micro-Bondapak CN column. The 5-component mobile phase system utilized in this technique has been described in great detail previously. This technique is capable of separating the internal standard, WR 184,806, from the candidate antimalarial, WR 180,409, as well as separation from most of the remaining interference. The procedure is illustrated more

clearly in the following flow-chart.



c. Results:

Determining the effectiveness of the system's ability to analyze WR 180,409 can best be accomplished by the analysis of

unknown samples. The unknown samples were prepared by the addition of known quantities of the candidate antimalarial, WR 180,409, to weighed quantities of blood obtained from the blood bank. The unknown samples were prepared to span a range of 100 ng of WR 180,409 per gram of blood to 1800 ng per gram of blood. Seven blood samples were studied in each of five trials. The results of the analyses of the unknowns by utilization of this system are shown in Table 8. The average error or average percent difference of the true value and the value found by utilization of this technique is approximately $\pm 8\%$.

A preliminary study was undertaken to determine the time course of the absorption and elimination of the candidate anti-malarial, WR 180,409, from tablets and capsules in four human volunteers. A 750 mg dose of WR 180,409 was presented in either 3 tablets or 3 capsules to each of four volunteers. Blood samples were withdrawn from these volunteers at various times and the corresponding blood level at these intervals was determined through the utilization of the HPLC technique. The blood levels obtained in these volunteers are shown in Table 9.

d. Discussion:

This technique for the analysis of WR 180,409 in blood samples is indeed cumbersome and somewhat complicated. Nevertheless, the results of the analysis of five series of unknowns demonstrates that this system is effective and may be acceptable for the analysis of blood samples obtained from human volunteers. Although this system can be utilized in its present form as has been demonstrated in the analysis of blood samples from four volunteer patients, it is anticipated that further improvements and simplifications will be made. Further improvements in the extraction and refinement procedure are still the primary goal of the current HPLC system. Utilization of a different internal standard, perhaps a derivative of WR 180,409 more closely resembling that compound in structure, may aid in the extraction technique and may function more efficiently in the reverse phase chromatographic procedure.

4. Development of an in vitro method for the determination of methemoglobin formation induced by a drug.

a. Background:

The 28 day toxicity studies of WR 225,448-succinate in dogs showed methemoglobinemia occurred 1-2 wk after daily oral dosage of 1, 3 or 9 mg/kg/day of the drug (Lee, 1979). Although the methemoglobinemia was seen 1-2 wk after the start

of dosing, it is not yet known whether the parent drug (unchanged WR 225,448) and/or its metabolite(s) caused the effect. It is difficult to obtain sufficient amount of purified metabolite(s) from either the in vivo study or an in vitro isolated organ metabolism study. The existing clinical methods for quantitating methemoglobin in plasma and/or whole blood involve chemicals that may form a complex with the test drug, thereby interfering with the assay. Therefore, the existing clinical methods for determining methemoglobin cannot be used for in vitro screening of drugs or chemicals to determine possible methemoglobin formation in human blood.

The objective of this study is to develop an in vitro screening method for the Army Drug Development Program as a way to screen candidate drugs for their potential to effect methemoglobin formation in human blood.

b. Materials and methods:

i. Preparation of hemoglobin solution:

Fresh heparinized human blood (10-20 ml) was centrifuged at 2,000 RPM for 10 min and the plasma was withdrawn from the centrifuge tube and discarded. The red blood cells (RBC) were suspended in twice the cell volume with 0.9% saline. The suspension was mixed well and centrifuged at 2,000 RPM for 10 min. The supernatant was discarded. This washing of the RBC was repeated 3 times. After the final wash the RBC were hemolyzed with twice the cell volume of deionized-distilled water. After mixing the solution with a Vortex mixer, 1 ml of toluene was added and the solution was mixed again. The hemolysate was centrifuged at 10,000 RPM for 15 min. The toluene layer on the top was completely removed and the remaining hemoglobin solution was relatively free of particulate matter and lipids. Without further dilution, this stock hemoglobin solution can be stored in a refrigerator for at least one month. The final hemoglobin solution used for the experiment was prepared by diluting 3 ml of the stock solution with deionized-distilled water to 100 ml in a 100 ml volumetric flask.

ii. Preparation of drug solutions:

Stock solutions (3×10^{-2} M) of sodium nitrite, primaquine diphosphate and WR 225,448 succinate in deionized-distilled water were prepared. Working solutions of each drug (3×10^{-3} M, 3×10^{-4} M and 3×10^{-5} M) were prepared by serial dilutions.

iii. Spectrophotometric determination of the concentration of hemoglobin (Hb) and methemoglobin (MHb):

A Varian Cary 219 spectrophotometer was used. An appropriate volume of the test drug solution was used such that the volume of the drug solution added to the appropriate volume of the Hb solution would give a final drug concentration of 10^{-3} M, 10^{-4} M, 10^{-5} M, 2×10^{-3} M, etc. Prior to each determination, a control Hb sample was also made with the same volume of Hb solution plus a volume of deionized-distilled water equal to the volume of the drug solution used. To illustrate, when the desired test drug concentration was 10^{-3} M, the control Hb solution was made by adding 2.9 ml of Hb solution and 0.1 ml of deionized-distilled water in a cuvet (10 mm pathlength). The test drug cuvet contained 2.9 ml of Hb solution plus 0.1 ml of 3×10^{-2} M drug solution. Three ml of deionized-distilled water was used as a blank for the control Hb and 0.1 ml of the 3×10^{-2} M drug solution plus 2.9 ml deionized-distilled water was used as a blank for the test sample containing 10^{-3} M of the test drug. Before each determination deionized-distilled water vs. deionized-distilled water was used for zero baseline adjustment.

The control Hb sample was scanned first from 600 nm to 500 nm (range = 1.0; gain = 0.25; chart display = 10; scan rate = 0.5 nm/sec; period = 1.0 sec; spectral band width = 0.5 nm). The chart and wavelength were returned to the 600 nm position and zero baseline was checked and re-adjusted if necessary before the test sample was scanned.

The concentration of Hb, MHb, % MHb and the net change of % MHb were calculated by the following formula:

$$[\text{Hb}] = \frac{(1.64)(\text{OD}_{576}) - (0.64)(\text{OD}_{560}) - (0.72)(\text{OD}_{540})}{10^4}$$

$$[\text{MHb}] = \frac{(\text{OD}_{540}) - (0.20)(\text{OD}_{560}) - (0.82)(\text{OD}_{576})}{0.20 \times 10^4}$$

$$[\text{C}]_T = [\text{Hb}] + [\text{MHb}]$$

$$\% [\text{MHb}] = \frac{[\text{MHb}]}{[\text{C}]_T} \times 100$$

$$\Delta \% \text{MHB} = \% [\text{MHb}]_{\text{Drug}} - \% [\text{MHb}]_{\text{Control}}$$

Where $[Hb]$, $[MHb]$ and $[C]_T$ are the concentrations of Hb, MHb and total contents respectively in either the control sample or the test sample. The numbers 1.64, 0.64 and 0.72 are the extinction coefficients of optical densities at 576 nm, 560 nm and 540 nm, respectively, for Hb. The numbers 0.20 and 0.82 are the extinction coefficients of the optical densities at 560 nm and 576 nm, respectively, for MHb (Benesch et al., 1965). All optical densities refer to a 10 mm light path.

c. Results:

The effects of sodium nitrite ($NaNO_2$), primaquine diphosphate and WR 225,448-succinate on the spectral changes of human Hb are summarized in Table 10. Table 11 summarizes the effect of these agents on MHb formation. Sodium nitrite at a concentration of 10^{-4} M produced a 2.3% MHb formation while at 10^{-3} M it caused 100% methemoglobin formation in the sample. Since the sample itself originally contained 7.4% MHb (determined by measuring % MHb of the control sample), the net MHb formation was 92.6%.

Primaquine diphosphate at a concentration of 10^{-4} M did not cause any MHb formation in human Hb. However, it caused 8.5%, 29.7%, 42.0% and 91.3% at concentrations of 10^{-3} M, 2×10^{-3} M, 3×10^{-3} M and 4×10^{-3} M respectively.

WR 225,448-succinate at 10^{-4} M did not cause any MHb formation in human Hb. It caused 22.8% MHb formation in human Hb at 10^{-3} M, which is higher than primaquine diphosphate at the same molar concentration but far less than that of sodium nitrite.

d. Discussion:

Available clinical methods for evaluating methemoglobinemia caused by drugs or chemicals are based on the characteristic that the methemoglobin absorption band at 630 nm is almost completely abolished by the addition of potassium cyanide, which converts the pigment to cyanmethemoglobin. The resulting change in absorbance is directly proportional to the concentration of methemoglobin in the solution (Dubowski, 1964). These clinical methods require careful handling of the blood specimens and prompt analysis. Delayed analysis, incomplete hemolysis and cloudy blood dilutions are potential sources of error, in addition to instrumental to other technical errors.

In our preliminary studies, we found that two major factors prevented us from using the clinical methods for the determination of methemoglobin formation caused by a drug

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in vitro. The first, a minor factor, is the time factor. The small amount of time allowed for performing the analysis after drawing fresh human blood is not sufficient for an in vitro screening of a series of concentrations of a drug. The major factor is that the chemicals involved in the clinical methods, such as potassium cyanide, form a complex with our test drugs which interferes with the analysis. This makes the clinical methods completely useless for the in vitro screening of the effect of a drug on methemoglobin formation in human blood.

The results of this study show that the method developed, as described in section b, is a simple, rapid, reliable and reproducible procedure to determine whether a drug or its metabolite (if obtainable) is a methemoglobin-producing agent in human blood in vitro. This method can be used to screen a number of drugs in a relatively short period of time.

The major time consuming part of the procedure was the preparation of the Hb solution. However, once the stock Hb solution was prepared, it can be stored in the refrigerator for at least one month. This may be due to the fact that the Hb solution was prepared with deionized-distilled water instead of phosphate buffer and that the relatively concentrated Hb itself was stable because it was relatively free of lipids and cell debris. Even the diluted Hb solution was stable during the entire experiment and for at least 6 hr at room temperature. Evidence for this is that the spectral curve between 600 nm and 500 nm for the control Hb remained unchanged and that the calculated [Hb] for the control Hb remained at a range of 4.73×10^{-5} to 5.00×10^{-5} throughout the experiment. Furthermore, each week for a month a sample of the stock Hb solution was taken, diluted and scanned from 600 nm to 500 nm as described above. It was found that both the spectral curve and the Hb concentration were essentially unchanged.

There are limitations to this in vitro method. For a drug to be scanned for its MHB formation potential, the drug must be relatively water soluble (3×10^{-4} M to 3×10^{-3} M solutions can be made) and the absorption peaks of the drug cannot be the same as those of the human hemoglobin.

5. The tissue distribution of DL-threo- α -(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol hydrochloride (WR 177,602·HCl) in mice.

a. Background:

The quinolinemethanol derivative, WR 177,602, is a diastereoisomer of mefloquine. WR 177,602 is 27 times more potent than quinine against Plasmodium berghei malaria infections when administered orally to the mouse. This compound is also effective in curing human and animal multidrug-resistant strains of malaria. The objective of this study was to determine the tissue distribution of this compound in mice.

b. Materials and methods:

Radioactive WR 177,602·HCl (Sp Act 32.3427 μ Ci/mg; Lot No. 469a-3-1) was synthesized by Monsanto Research Corp. (Dayton, OH) with the 14 C-label in the alcohol carbon. The nonradioactive compound (Lot AD, Bottle No. BE 77728) was prepared by Cordova Chemical Co. (Sacramento, CA). Radioactive and nonradioactive compounds were codissolved to obtain a suitable specific activity of 1.62 μ Ci/mg. Radiochemical purity of the compound was assayed by thin-layer chromatography. A suspension of the resulting mixture of WR 177,602- 14 C·HCl was prepared in 0.2% methylcellulose and 0.4% Tween® 80 in deionized water, 1 mg/ml. A premixed liquid scintillation solution, Hydromix® (Yorktown Research Co., S. Hackensack, NJ) was used for radioassay. All other chemicals and solvents used were reagent grade quality.

Albino, ICR female mice from the Walter Reed Colony [Walter Reed Medical (Institute of Cancer Research) Barrier-Reared mice], weighing about 25 gm, were used. The mice were fed D and G Laboratory diet (G.L. Baking Co., Frederick, MD), and were maintained in a temperature controlled room with a 12-hr light-dark cycle. The mice were fasted for about 18 hr prior to dosing but were permitted water ad libitum. A dose of 10 mg/kg was administered to each mouse by oral intubation. Seven groups of 3 mice each were housed in standard rodent cages. All groups were allowed water ad libitum, but food was not allowed until 4 hr after dosing. Standard doses were taken for radioassay before, during and after dosing the animals.

At the chosen sampling times (12, 24, 36, 48, 60, 72 and 96 hr after dosing) the 3 animals in a group were anesthetized with ether, exsanguinated via transection of the surgically exposed femoral vessels and various whole organs and tissue samples were excised. Each whole organ sample and tissue sample for each time interval group represents a pooled sample from the 3 mice assigned to that group. Each pooled

sample was placed in a preweighed container, weighed, and sufficient absolute methanol was added to cover the tissues. The respective groups of carcasses were frozen for later processing.

Each organ/tissue pool was homogenized in absolute methanol in a Waring® blender and the homogenate was slurried into a glass column. After the methanol was eluted from the homogenate and the eluate was recycled through the column, extraction was accomplished by slowly passing 3-4 bed volumes of additional methanol through the column. The total volume of these combined eluates from each organ/tissue sample was measured and aliquots were taken for radioassay. The methanol extracts were evaporated to dryness in a flash evaporator at 32°C and were reconstituted in small amounts of absolute methanol. Aliquots were taken for radioassay and the samples stored in a freezer at -10°C for further studies.

The 3 individual carcasses from each respective group were defrosted at room temperature and homogenized together in absolute methanol in a tissue grinder (Model SD45, Tekmar Co., Cincinnati, OH) and the homogenates processed in the same manner as the tissue samples.

Fifteen ml of premixed scintillation fluid (Hydromix®) were added to each aliquot taken for radioassay. The samples were counted for 10 min or 10,000 counts in a Searle Mark II liquid scintillation counter. Quenching and counting efficiency were corrected by external standardization.

Appropriate amounts of the reconstituted samples were streaked on EM precoated silica gel G F254 TLC plates, 0.25 mm thickness (EM Laboratories, Elmsford, NY), and developed for 10 cm from the origin with ethylene dichloride:glacial acetic acid:methanol (8:1:1, v/v/v) as the solvent system. This solvent was the best among seven TLC solvent systems tested prior to the study. After air-drying, the plates were visualized with ultraviolet light (254 nm) and scanned with a Varian Model 6000 Radioscanner with integrator (time constant, 1 sec; speed, 10 cm/hr; attenuation, 10 cps). The integration of the radioscanner provided a means for measuring the relative percentage of radioactivity for each peak. A control streak of ¹⁴C-WR 177,602-HCl was placed on each plate as an R_f comparison standard.

c. Results:

The distribution, concentration and elimination of ^{14}C -WR 177,602 were studied in selected organs and tissues at 12, 24, 36, 48, 60, 72 and 96 hr after oral dosing in female mice. In this experiment, elimination of drug-derived radioactivity via urinary and fecal routes was not concurrently determined. Therefore, total dose recovery cannot be calculated for each group.

The sites of distribution (excluding the gastrointestinal tract and its contents) of ^{14}C WR 177,602-derived radioactivity are shown in Table 12. At 12 and 24 hr, respectively, after dosing the major sites are the lungs (3.0% and 1.6%), liver (7.2% and 4.8%), kidneys (1.6% and 1.1%) and the residual carcasses (21.2% and 13.2%). At 36 hr after dosing the liver (2.8%), lungs (1.0%) and residual carcasses (7.0%) still contain relatively high levels of drug-derived radioactivity. All other organs/tissues contained less than 1% of the total radioactivity at 12 hr with uniformly declining values through 96 hr.

The major organs/tissues (excluding the gut) concentrating the drug-derived radioactivity on a μg ^{14}C -drug equivalents per gm tissue basis (Table 12) were: at 12 hr after dosing, the gall bladder plus bile (269.1), lungs (60.9), abdominal fat (15.2), kidney (13.7), liver (12.6) and adrenal glands (9.9); at 24 hr after dosing, gall bladder plus bile (23.1), lungs (22.9), eyes and harderian glands (12.7), kidney (8.9) and liver (8.2). All other tissues throughout the course of this study contained concentrations less than 8 $\mu\text{g/g}$ and declined uniformly through 96 hr. The time course characteristics of the decline of drug-derived radioactivity are indicated in Table 13, which shows the biological half life of elimination ($t_{1/2}$) of ^{14}C -drug-derived radioactivity for selected organs and tissues. The $t_{1/2}$ for whole blood (20.30 hr, $R^2 = .990$) and plasma (19.45 hr, $R^2 = .994$) are reported elsewhere and are stated here for comparison.

Of the organs/tissues containing sufficient drug-derived radioactivity for analysis, the major sites (excluding gut) concentrating parent drug on a μg drug/gm basis (Table 14) were: at 12 hr after dosing, the lungs (39.2), kidney (8.5), liver (7.5), submaxillary salivary glands (6.5) and heart (5.0); at 24 hr after dosing, lungs (9.9), eyes (8.0), liver (4.3) and kidney (4.2). The lungs contained a high concentration of parent drug with 9.5, 4.6 and 2.6 $\mu\text{g/gm}$ at 36, 48 and 60 hr after dosing, respectively. Kidney (2.3, 2.3 and 0.4 $\mu\text{g/gm}$) and

liver (2.7, 1.6 and 0.9 $\mu\text{g/gm}$) also contained relatively high concentrations of parent drug at these respective times after dosing. The biological half life of elimination ($t_{1/2}$) of parent drug was calculated similarly to those in Table 13 and was: 13.34 hr ($R^2 = .851$) for lungs; 16.18 hr ($R^2 = .999$) for liver; and 12.28 hr ($R^2 = .951$) for kidney. The $t_{1/2}$ for parent drug in plasma (16.06 hr, $R^2 = .979$) is reported elsewhere and is stated here for comparison.

d. Discussion:

The tissue distribution and concentration of WR 177,602·HCl was investigated in female mice after oral administration. A high percentage of the administered dose was found in the liver, lungs, kidneys and residual carcasses (as well as the GI tract) of these animals over the course of the study. The concentration of ^{14}C -drug equivalents per gram tissue, as well as the concentration of parent drug-associated radioactivity, also remained high in these organs (and carcasses) over the course of the study. The biological half life of elimination of ^{14}C -drug equivalent radioactivity and parent drug associated radioactivity in these tissues apparently follow a monoexponential decline over the time course studied. The interpretation of this with regard to ^{14}C -drug equivalent data is difficult since such concentration values represent a mixture of substances (parent drug and metabolites) and, therefore, the resulting governing model equation is not necessarily linear with respect to time (i.e., $\ln([\text{parent}]_t + [\text{metabolite 1}]_t \dots + [\text{metabolite n}]_t) = \ln([\text{parent}]_0 e^{-k_p t} + [\text{metabolite 1}]_0 e^{-k_{m1} t} \dots + [\text{metabolite n}]_0 e^{-k_{mn} t})$ is not linear). However, coupled with the information that the parent drug appears to follow the same monoexponential clearance (elimination) process(es), several possibilities exist for explaining the apparent monoexponential tissue clearance of both total radioactivity and parent drug concentrations: first, if the rate constants for the tissue clearance (elimination) of the parent drug and metabolites are equal, then in characterizing the sum, we have characterized the parts; and, secondly, if the rate constants of several components were large (fast), resulting in negligibly small exponential terms, the $t_{1/2}$ measurement would represent the characteristics of the slow component. It is impossible to characterize the true kinetics of this drug in mice further at this time without thorough, sensitive analysis of the metabolite components of the system. A further complicating factor may be due to possible biliary excretion and/or enterohepatic circulation of this drug, inasmuch as persistently high concentrations of ^{14}C -drug equivalents (and parent drug in the case of liver)

are found in the gall bladder and bile, and in the liver during the course of the study.

The ability of the ^{14}C -drug equivalents to concentrate in skeletal muscle and fat (and residual carcasses) gives some indication, along with previously reported data, that the drug may accumulate there during the first 24-36 hr, with clearance thereafter. The adrenal glands, submaxillary salivary glands, heart and spleen also concentrate ^{14}C -drug equivalents of this drug.

6. Development of new antimalarial drugs.

a. Background:

The Department of Pharmacology is also charged with the responsibility of writing Notice of Claimed Investigational Exemption for New Drug (IND) submissions. These include planning and designing the experiments, and assembling, evaluating, coordinating and correlating the data required for both the initial submission and all supplementary submissions for each drug. The data must be continuously monitored and evaluated from both in-house and contract sources, as well as proprietary and open literature sources.

b. Current Investigational New Drugs:

Fourteen IND's are currently classified in the Active status. These include two combinations and the remainder are single drugs. In addition three new drugs have been elevated to IND status: WR 225,448; WR 226,253; and WR 228,258.

c. Technical monitoring of contracts necessary for data generation:

Thirteen active contracts were closely guided by the Department. These ranged from pharmacological areas such as toxicology and drug metabolism to those of drug formulation and development of methods to determine blood levels of drugs.

As an integral part of the task, specifications for three RFQ's were written and appropriate Selection Boards convened.

Table 1

Extraction by Ethyl Acetate of Approximately Equal Concentrations
(1000 ng/ml) of WR 180,409 and WR 184,806 at Various pH

pH	Percent Extracted		Quality of Chromatographic Peaks
	WR 180,409	WR 184,806	
2.0	undetermined undetermined	23.8 undetermined	Poor; significant interference
4.1	72.5	70.8	Poor; tailing peaks; baseline interference
6.0	87.6	84.8	Poor; tailing peaks; baseline interference
7.4	90.8	91.6	Adequate separation
	94.7	93.9	Some baseline interference; slight tailing of peaks
8.2	77.8	92.5	Tailing peaks; near baseline separation
	84.8	90.1	"
10.1	75.0	98.6	"
	64.6	94.6	"
12.0	53.6	97.6	"
	60.6	98.3	"
13.0	54.3	96.5	"
	57.1	97.4	"

Table 2

Recovery of WR 180,409 and WR 184,806 Extracted with Various Organic Solvents from Spiked Blood
Equilibrated with pH 7.4 Phosphate Buffer

Organic Solvent	% Recovery ^a		Chromatographic Appearance
	WR 180,409	WR 184,806	
Ethyl Acetate	96.7 \pm 9.8	108.0 \pm 20.5	Variable - clean separation to marked interference (n = 11)
Ethyl Ether	82.9	90.4	Minor baseline interference pattern
Isopropyl Acetate	109.0 107.0	133.0 130.0	Good peak separation with some baseline interference
t-Butyl Acetate	51.3	100.4	Good; clean chromatogram and baseline separation
50% t-Butyl Acetate/ 50% Ethyl Acetate	86.8	103.2	Fair; clean, minor baseline interference
Amyl Acetate	--	--	Very poor; interference pattern too great to quantitate
Methyl Isobutyl Ketone	42.0	62.2	Poor; marked baseline interference
Methyl Ethyl Ketone	--	--	Poor; interference pattern too great to quantitate

Table 2 (Continued)

Organic Solvent	% Recovery ^a		Chromatographic Appearance
	WR 180,409	WR 184,806	
Sec Butanol	--	--	Poor; interference pattern too great to quantitate
n-Amyl Alcohol	--	--	Poor; interference pattern too great to quantitate
Octanol	--	--	Solvent cannot be evaporated
Xylene	38.7	59.5	Good baseline separation
Toluene	51.1	64.8	Some baseline interference
Iso Octane	8.4	14.8	Good baseline separation
Hexane	13.8	20.8	Good baseline separation
50% Hexane + 50% Ethylene Dichloride	54.4	74.6	Baseline separation with minimal interference
Hexane + 1% Ethanol	12.7	21.4	Good separation
Hexane + 9% Ethanol	31.6	35.3	Minimal baseline interference
Hexane + 16.6% Ethanol	42.5	39.7	Significant interference
Hexane + 1% Octanol	10.2	30.3	Slight baseline interference

Table 2 (Concluded)

Organic Solvent	% Recovery ^a		Chromatographic Appearance
	WR 180,409	WR 184,806	
Hexane + 1% Butanol	21.9	32.9	Good baseline separation
Hexane + 9% Butanol	74.8	83.4	Marked baseline interference
Hexane + 16.6% Butanol	74.2	87.1	Marked baseline interference
Hexane + 33.3% Butanol	--	84.5	WR 180,409 cannot be determined Marked baseline interference
Cyclohexane	16.1	24.9	Good baseline separation
Cyclohexane + 1% Butanol	27.6	37.6	Minor baseline interference
Cyclohexane + 9% Butanol	78.1	95.4	Significant baseline interference
Cyclohexane + 16.6% Butanol	61.5	80.5	Significant baseline interference
Chloroform	8.5	46.1	Minor baseline interference
10% Chloroform/ 90% Ethyl Ether	72.5	96.4	Minor baseline interference
Carbon Tetrachloride	19.5	70.5	Minor baseline interference

^aDetermined by a comparison of the HPLC peak of the extracted sample with the HPLC peak of the antimalarial standard dissolved in methanol.

Table 3

Recovery of WR 180,409 and WR 184,806 Extracted with Various Organic Solvents
from Spiked Blood Samples Equilibrated with 3 M Sodium Chloride

Organic Solvent	% Recovery ^a		Chromatographic Appearance
	WR 180,409	WR 184,806	
Octanol	--	--	Solvent can not be evaporated
Chloroform	27.6	63.9	Fair; baseline separation
Ethyl Acetate	88.6	92.8	Fair; baseline separation with some interference
Ethyl Ether	79.4	91.9	Baseline interference pattern
Iso Octane	14.3	28.1	Minimal baseline interference
Xylene	82.5	33.9	Poor; marked baseline interference
Toluene	66.5	88.8	Baseline interference

^a Determined by comparison of HPLC peak of extracted sample with HPLC peak of antimalarial standard dissolved in methanol.

Table 4

Recovery of WR 180,409 and WR 184,806 Extracted with Ethyl Acetate
from Spiked Blood Samples Equilibrated with pH 7.4 Phosphate Buffer

Trial #	Percent Recovery ^a	
	WR 180,409	WR 184,806
1	97.2	132.5
2	98.6	134.6
3	82.1	88.1
4	86.8	88.7
5	94.7	93.9
6	82.2	85.9
7	98.5	83.1
8	112.1	132.5
9	108.2	117.3
10	103.0	116.0
11	<u>100.0</u>	<u>115.0</u>
$\bar{X} \pm \text{s.d.} = 96.7 \pm 9.8$		$\bar{X} \pm \text{s.d.} = 108.0 \pm 20.5$

^aDetermined by comparison of HPLC peak of extracted sample with HPLC peak of antimalarial standard dissolved in methanol.

Table 5
WR 180,409 and WR 184,806 Extraction by and Recovery from Silica SEP-PAK

Solvent System	Ethyl Acetate			Dimethylformamide			Ethylene Dichloride		
	WR 180,409	WR 184,806	Chromatogram ^a	WR 180,409	WR 184,806	Chromatogram ^a	WR 180,409	WR 184,806	Chromatogram ^a
Control Sample	100% 100%	100% 100%	Poor Poor	100% 100%	100% 100%	Poor Poor	100% 100%	100% 100%	Poor Poor
Sample Not Adsorbed	0 0	0 0	Clear Clear	C.D. ~100%	C.D. ~100%	Very poor Very poor	0 0	0 0	Clear Clear
Solvent Wash	0 0	0 0	Clear Clear	0 0	0 0	Fair Fair	0 0	0 0	Clear Clear
Hexane	0 0	0 0	Clear Clear	- -	- -	Not used Not used	- -	- -	Not used Not used
Ethylene Dichloride	0 0	0 0	Clear Clear	0 0	0 0	Clear Clear	- -	- -	Not used Not used
First Methanol Elution	~90% ~88%	C.D. ~13%	Very poor Very poor	C.D. C.D.	C.D. C.D.	Very poor Very poor	40% 40%	43% 37%	Poor Poor
Second Methanol Elution	0 0	16% 17%	Fair Fair	0 0	0 0	Poor Poor	16% 19%	29% 25%	Fair Fair
Third Methanol Elution	0 0	3% 0	Clear Clear	0 0	0 0	Clear Clear	7% 6%	27% 22%	Clear Clear
Warm Methanol	0 0	0 0	Poor Poor	0 0	0 0	Clear Clear	0 0	10% 8%	Clear Clear
Methanol/Water	0 0	0 0	Clear Clear	0 0	0 0	Clear Clear	0 4%	11% 14%	Clear Clear
Dilute HCl	0 0	0 0	Clear Clear	- -	- -	Not used Not used	- -	- -	Not used Not used

^a Clear = no interference in area of drug peak; C.D. = could not be determined.

Table 6
Effects of Pre-Extraction on the Recovery and Purity of WP 180,409 and WP 184,806

Acid	Organic Solvent	Pre-Extraction		Base	Organic Solvent	Extraction		HPLC Chromatogram
		% Loss	% Recovery					
		WR 180,409	WR 184,806			WR 180,409	WR 184,806	
HCl	Ethyl Acetate (10 ml) 2 Extractions	74 56	64 49	KOH	Ethyl Acetate	14 24	24 33	Good baseline separation Good baseline separation
HCl	Ethyl Ether (10 ml) 2 Extractions	39 31	18 15	KOH	Ethyl Acetate	33 42	77 82	Poor baseline interference
HCl	Hexane (10 ml) 2 Extractions	0 0	0 0	KOH	Ethyl Acetate	78 82	100 105	Improved baseline Improved baseline
HCl	Iso-Octane (10 ml) 2 Extractions	0 0	0 0	KOH	Ethyl Acetate	80 77	69 69	Improved baseline
H ₂ SO ₄	Hexane (10 ml) 2 Extractions	6 9	5 10	KOH	Ethyl Acetate	70 84	100 94	No improvement No improvement
H ₂ SO ₄	Hexane (10 ml) 2 Extractions	8 9	11 10	KOH	Ethyl Acetate	81 84	93 95	No improvement No improvement
H ₂ SO ₄	Iso-Octane (10 ml) 2 Extractions	5 8	6 4	KOH	Ethyl Acetate	88 79	102 95	No improvement No improvement
H ₂ SO ₄	Heptane (10 ml) 2 Extractions	7 7	10 10	KOH	Ethyl Acetate	80 94	77 89	No improvement No improvement
H ₂ SO ₄	Xylene	34	58	KOH	Ethyl Acetate	43	46	No improvement

Table 7
Apparent Solvent Interaction with the Antimalarial Agents WR 184,806 and WR 180,409^a

Initial Solvent Mixture	Trial A	Trial B	Trial C
1. WR 180,409 and WR 184,806 in 50 μ l methanol mixed with 5 ml ethyl acetate; shake for 30 min, evaporate for 20 hr at 40°C	Redissolved in mobile phase of 50% hexane/50% ethylene dichloride; large solvent front interference WR 184,806 = 13.7% of control WR 180,409 = 2.0% of control	Heat mixture in Trial A to 50°C, sonicate for 30 min; large solvent front interference WR 184,806 = 19.2% WR 180,409 = 4.3%	Evaporate solvent from Trial B; redissolve in methanol, large solvent front interference remains WR 184,806 = 31.6% WR 180,409 = 14.4%
2. Repeat #1 but do not shake in order to maintain minimal contact with glass	Redissolve in mobile phase of 50% hexane/50% ethylene dichloride; large solvent front interference. No drug peaks	Heat mixture in Trial A to 50°C, sonicate for 35 min; large solvent front interference WR 184,806 = 11.5% WR 180,409 = 2.0%	Evaporate solvent from Trial B; redissolve in methanol, large solvent front interference remains WR 184,806 = 28.8% WR 180,409 = 5.8%
3. WR 180,409 in 50 μ l methanol mixed with 5 ml ethyl acetate with minimal shaking to avoid contact with glass; evaporate for 20 hr at 40°C	Redissolved in mobile phase as in 1 and 2; solvent front interference. No drug peak >1%	Heat mixture in Trial A to 50°C, sonicate for 45 min; solvent front interference remains, slightly more drug <3%	--
4. WR 184,806 - repeat in same manner as #3	Redissolved in mobile phase as in 1 and 2; solvent front interference; no drug peak	Heat mixture in Trial A same as in 3B; solvent front interference remains; no drug peak	--

Table 7 (Continued)

Initial Solvent Mixture	Trial A	Trial B	Trial C
5. 50 μ l methanol in 5 ml ethyl acetate evaporated to dryness in 30 min	Redissolved in mobile phase as in 1 and 2; no solvent front interference	Heat mixture in Trial A to 50°C and sonicate for 85 min; no solvent front interference	Evaporate overnight at 40°C, redissolve in methanol; no solvent front interference
6. 50 μ l of methanol containing WR 180,409 and WR 184,806 evaporated to dryness in the bottom of a conical tube	Redissolved in mobile phase of 50% hexane/50% ethylene dichloride; no solvent interference pattern; no drug peaks		
7. 50 μ l of methanol containing WR 180,409 and WR 184,806 mixed with 10 ml ethyl ether for 72 hr, evaporated to dryness	Redissolved in mobile phase of 50% hexane/50% ethylene dichloride; solvent front interference + baseline interference WR 184,806 = 61.3% of control WR 180,409 = 13.3% of control		
8. Repeat #7 with hexane as solvent	Repeat redissolution in mobile phase as in #7; small solvent front interference WR 184,806 = 60.0% of control WR 180,409 = 28.9% of control		
9. Repeat #7 with ethylene dichloride as solvent	Repeat redissolution in mobile phase as in #7; small solvent front interference WR 184,806 = 74.0% of control WR 180,409 = 31.6% of control		

Table 7 (Concluded)

Initial Solvent Mixture	Trial A	Trial B	Trial C
10. Repeat #7 with xylene as solvent	Repeat redissolution in mobile phase as in #7; significant interference pattern with good recovery of the antimalarials WR 184,806 = 93.4% of control WR 180,409 = 61.5% of control		
11. Repeat #7 with 10% methanol/90% xylene mixture as solvent	Repeat redissolution in mobile phase as in #7; marked interference pattern with good recovery of the antimalarials WR 184,806 = 90.7% of control WR 180,409 = 75.6% of control		

^aPercent of Control = area of the chromatographed antimalarial peak compared to the antimalarial standard chromatographed from methanol.

Table 8
HPLC Analysis of WR 180,409 in Blood Samples

Tube	Trial I			Trial II			Trial III			Trial IV			Trial V		
	Actual (ug/g)	Found (ug/g)	% Diff	Actual (ug/g)	Found (ug/g)	% Diff	Actual (ug/g)	Found (ug/g)	% Diff	Actual (ug/g)	Found (ug/g)	% Diff	Actual (ug/g)	Found (ug/g)	% Diff
1	0.432	0.463	+7.2	0.605	0.606	+0.2	0.076	0.156	105.3	0.307	0.324	+5.5	0.161	0.166	+3.1
2	1.085	1.037	-4.4	0.309	0.353	+14.2	0.447	0.446	-0.2	0.552	0.541	-1.9	0.552	0.549	-0.5
3	0.764	0.689	-9.8	0.076	0.091	+20.2	0.891	0.917	+2.9	0.812	0.768	-5.4	0.924	0.886	-4.1
4	0.110	0.103	-6.3	0.891	0.889	-0.2	1.281	1.252	-2.3	0.735	0.736	+0.1	0.812	0.788	-2.9
5	0.110	0.106	-3.6	1.155	1.176	+1.8	0.762	0.821	+7.7	1.587	1.424	-10.3	1.587	1.532	-3.5
6	1.518	1.511	-0.5	1.451	1.331	-8.3	1.155	1.241	+7.4	0.161	0.161	0.0	1.349	1.275	-5.5
7	1.757	1.590	-9.5	0.447	0.426	-4.7	0.153	0.144	-5.9	1.045	1.137	+8.8	0.122	0.138	+13.1

Table 9

Blood Levels of WR 180,409 in Four Human Volunteers Following
Oral Administration of 750 mg of WR 180,409

Time (hr)	Capsules Lot E-556		Tablets Lot D-522	
	Vol #345 ($\mu\text{g/g}$)	Vol #347 ($\mu\text{g/g}$)	Vol #346 ($\mu\text{g/g}$)	Vol #348 ($\mu\text{g/g}$)
-0.25	0.000	0.000	0.000	0.000
1.0	0.119	0.000	0.000	0.000
2	0.230	0.190	0.188	0.000
4	0.349	0.323	0.270	0.175
6	0.391	0.370	- ^a	0.222
8	0.411	0.523	0.311	0.263
12	0.365	0.524	0.229	0.239
16	0.412	0.465	0.325	0.281
20	0.320	0.455	0.251	0.269
24	0.341	0.456	0.222	0.190
28	0.458	0.373	0.266	0.142
30	0.327	0.479	0.241	0.222
36	0.333	0.427	0.196	0.306
40	0.345	0.423	0.150	0.282
44	0.249	0.265	0.299	0.230
48	0.343	0.316	0.198	0.199
60	0.334	0.398	0.200	0.189
72	0.234	0.321	0.122	0.142
96-98	0.279	0.275	0.104	0.151
144-146	0.250	0.196	0.070	0.105
168-170	0.206	0.182	0.061	0.070
312-314	0.136	- ^a	0.008	0.004
480-482	0.096	0.077	0.000	0.005

^aNot run.

Table 10

The Effect of Sodium Nitrite, Primaquine Diphosphate and
WR 225,448-Succinate on the Spectral Changes of Human Hemoglobin

Sample	O.D.		
	540 nm	560 nm	576 nm
Control Hb	0.760	0.460	0.805
NaNO ₂ 10 ⁻⁴ M	0.700	0.440	0.735
Control Hb	0.760	0.460	0.805
NaNO ₂ 10 ⁻³ M	0.400	0.290	0.275
Control Hb	0.760	0.460	0.805
Primaquine 10 ⁻⁴ M	0.705	0.430	0.735
Control Hb	0.730	0.445	0.770
Primaquine 2 x 10 ⁻³ M	0.550	0.380	0.550
Control Hb	0.705	0.425	0.740
Primaquine 3 x 10 ⁻³ M	0.430	0.329	0.410
Control Hb	0.682	0.418	0.720
Primaquine 4 x 10 ⁻³ M	0.330	0.280	0.230
Control Hb	0.760	0.460	0.801
WR 225,448 10 ⁻⁴ M	0.760	0.460	0.800
Control Hb	0.760	0.460	0.801
WR 225,448 10 ⁻³ M	0.720	0.470	0.720

Table 11
The Effect of Sodium Nitrite, Primaquine Diposphate and
WR 225,448-Succinate on Methemoglobin Formation in Human Hemoglobin

Sample	[Hb]	[MHb]	% MHb	Δ %MHb
Control Hb	5.00×10^{-5}	0.40×10^{-5}	7.4	
NaNO ₂ 10^{-4} M	4.19×10^{-5}	0.45×10^{-5}	9.7	2.3
Control Hb	5.00×10^{-5}	0.40×10^{-5}	7.4	
NaNO ₂ 10^{-3} M	0	5.8×10^{-5}	100.0	92.6
Control Hb	5.00×10^{-5}	0.40×10^{-5}	7.4	
Primaquine 10^{-4} M	5.00×10^{-5}	0.40×10^{-5}	7.4	0
Control Hb	5.00×10^{-5}	0.40×10^{-5}	7.4	
Primaquine 10^{-3} M	4.22×10^{-5}	0.80×10^{-5}	15.9	8.5
Control Hb	4.50×10^{-5}	0.50×10^{-5}	10.0	
Primaquine 2×10^{-3} M	2.63×10^{-5}	1.50×10^{-5}	39.7	29.7
Control Hb	4.30×10^{-5}	0.65×10^{-5}	13.0	
Primaquine 3×10^{-3} M	1.51×10^{-5}	1.40×10^{-5}	55.0	42.0
Control Hb	4.22×10^{-5}	0.40×10^{-5}	8.7	
Primaquine 4×10^{-3} M	0	3.80×10^{-5}	100.0	91.3

Table 11 (Concluded)

Sample	[Hb]	[MHB]	% MHB	Δ %MHB
Control Hb	4.73×10^{-5}	0.55×10^{-5}	10.4	
WR 225,448 10^{-4} M	4.73×10^{-5}	0.55×10^{-5}	10.4	0
Control Hb	4.73×10^{-5}	0.55×10^{-5}	10.4	
WR 225,448 $\times 10^{-3}$ M	3.62×10^{-5}	1.80×10^{-5}	33.2	22.8

Table 12
Tissue Distribution and Concentration of WR 177,602-¹⁴C-Derived Radioactivity in Orally Treated Female Mice

Organ/Tissue	Percent (%) Administered Dose ^a						
	12 hr	24 hr	36 hr	48 hr	60 hr	72 hr	96 hr
Eyes (harderian glands)	.09 (2.83)	.34 (12.71)	.20 (5.34)	.10 (3.27)	.06 (13.99)	.02 (.76)	.02 (.53)
Brain	.52 (2.84)	.30 (1.70)	.18 (1.68)	.11 (.57)	.07 (.31)	.02 (.10)	.04 (.37)
Salivary Glands (submaxillary)	.38 (7.27)	b	.16 (2.06)	.08 (1.27)	.06 (.80)	.03 (.52)	.01 (.16)
Heart	.34 (6.89)	.18 (3.61)	.13 (2.33)	.07 (1.39)	.04 (.80)	.02 (.49)	.02 (.35)
Lungs	3.05 (50.90)	1.56 (22.93)	1.00 (13.38)	.48 (7.62)	.27 (3.98)	.06 (1.00)	.03 (.46)
Gall Bladder	.07 (269.09)	.03 (23.12)	.03 (9.96)	.02 (11.75)	.01 (5.24)	<.01 (2.48)	<.01 (2.40)
Liver	7.20 (12.63)	4.80 (8.18)	2.78 (3.85)	1.81 (3.26)	.96 (1.77)	.49 (.87)	.27 (.48)
Spleen	.34 (6.75)	.20 (4.93)	.11 (2.25)	.05 (1.41)	.10 (2.67)	.01 (.22)	.01 (.38)
Kidney	1.63 (13.73)	1.09 (8.90)	.55 (3.89)	.44 (3.35)	.21 (1.57)	.09 (.77)	.05 (.37)
Adrenal Glands	.04 (5.88)	.02 (4.85)	.03 (4.27)	.01 (2.18)	.01 (1.65)	.01 (2.68)	<.01 (.31)
Abdominal Fat	c (15.18)	c (1.54)	c (1.25)	c (.57)	c (.45)	c (.26)	c (.13)
Skeletal Muscle	c (8.47)	c (.98)	c (1.56)	c (.45)	b	c (.11)	b
Residual Carcasses	21.22 (178.28)	13.16 (110.55)	7.01 (58.89)	3.86 (32.42)	2.15 (18.10)	1.02 (8.58)	.54 (4.50)
GI Tract plus contents	12.88 (9.86)	10.32 (8.40)	4.88 (3.70)	2.79 (2.13)	1.32 (1.11)	.67 (.48)	.21 (.14)

^aNumber in parentheses represents ug of ¹⁴C-drug equivalents per g tissue.

^bInsufficient radioactivity for analysis.

^cOnly small fraction of tissue mass taken; these tissue values along with bone, etc., are reflected in values for residual carcasses.

Table 13
Biological Half-Life of Elimination of ^{14}C -Drug-Derived Radioactivity
from Tissues of the Mouse after Oral Dosing with WR 177,602-HCl

Tissue	$\mu\text{g } ^{14}\text{C-drug Equiv/g Tissue vs. Time}$		
	$t_{1/2}^a$	R^2	Residual Error
Lungs	12.02	.995	1.9102
Liver	17.27	.987	.31397
Kidney	15.71	.985	.40351
Brain	20.64	.866	.16462
Salivary Glands	15.68	.988	.10987
Heart	15.95	.993	.04862
Gall Bladder	21.17	.875	9.5983
Spleen	18.01	.898	.710871
Adrenal Glands	19.84	.921	.92576
Abdominal Fat	19.39	.962	.01514
Residual Carcass	15.06	.995	24.881
GI Tract (plus contents)	13.05	.872	2.3691

^a $t_{1/2}$ values are calculated using the equation $t_{1/2} = \ln 2/\beta$, where β represents the first-order rate constant of elimination from the monoexponential regression equation: $C = C_0e^{-\beta t}$.

Table 14
Concentration of Parent Drug in Organs and Tissues
of Mice after Oral Dosing with ^{14}C -WR 177,602·HCl^a

Tissue	ug Parent Drug/g Tissue ^a				
	12 hr	24 hr	36 hr	48 hr	60 hr
Lungs	39.19 (77)	9.86 (43)	9.50 (71)	4.57 (60)	2.55 (64)
Liver	7.45 (59)	4.25 (52)	2.70 (70)	1.63 (50)	.92 (52)
Kidney	8.51 (62)	4.18 (47)	2.33 (60)	2.28 (68)	.39 (25)
GI Tract	5.13 (52)	5.54 (66)	1.85 (50)	.49 (23)	b
Eyes	1.33 (47)	8.01 (63)	.55 (14)	b	b
Brain	2.67 (94)	1.65 (97)	1.50 (89)	b	b
Salivary Glands	6.54 (90)	b	1.61 (78)	b	b
Heart	5.03 (73)	.94 (26)	b	b	b
Residual Carcasses	114.10 (64)	53.06 (48)	25.91 (44)	b	b

^aPercent of total radioactivity of the sample attributable to parent drug is shown in parentheses.

^bSample can not be analyzed.

Project 3M162770A803 DRUG DEVELOPMENT

Work Unit 087 Determination of pharmacological effects of anti-malarial drugs

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION*	2 DATE OF SUMMARY*	REPORT CONTROL SYMBOL	
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(U) Antimalarials (U) Pharmacokinetics (U) Drug Intolerance (U) Analytical Chemistry							
23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code)							
<p>23 (U) The technical objectives includes (1) The determination of biochemical indices that reliably predict drug intolerance in man. (2) The evaluation of effects of parasites and drugs on biochemical mechanisms as indices of prophylaxis and treatment. (3) The gas chromatographics-coupled mass spectrometric analysis of drugs and drug metabolites. (4) Analytical support for special and collaborative projects. The prediction of drug intolerance, the effects of drugs and infection on biochemical mechanisms and the understanding of drug and drug metabolite structure are relevant to the Army's malaria and tropical disease program.</p> <p>24 (U) Tissues and fluids from animals will be analyzed to determine metabolic processes affected by parasitic diseases and therapeutic and prophylactic drugs. Metabolic products, enzymes, isoenzymes, nucleic acids, lipids and proteins will be the primary focus. Methods will be developed and utilized to analyze biological specimens for compounds of interest.</p> <p>25 (U) 78 10-79 09 Studies were continued on the effects of antimalarials on metabolic and physiological processes and the metabolic pathways in parasites. Deamination and deribosylation of adenosine was found to occur on or at the surface of the limiting membrane of "free" malaria parasites. Method was developed for the determination of nucleic acids in the purine salvage pathway of malaria parasites. Chloroquine absorption and clearance in perfused pig livers was ascertained. Short term uptake and amino acid metabolism in promastigotes of <i>Leishmania braziliensis panamensis</i> was investigated. The analysis of drug and drug metabolites by mass spectrometry was continued. This task was terminated due to change in priorities. For technical report see WRAIR Annual Progress Report Oct 78 to 30 Sept 79</p>							

Project: 3M162770A803 DRUG DEVELOPMENT

Work Unit 088 Biochemical Research on Antimalarials

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The objective of this unit are the study of biochemical processes in malaria and other parasitic diseases, the biochemical effects of antimalarials, the biochemistry of host-parasite interactions in the presence and absence of drugs, and to define the metabolic processes in parasitemia susceptible to drug therapy. The speciation of biomolecules by mass spectrometry are reported.

1. The uptake of purine bases and nucleosides by normal rat erythrocytes, Plasmodium berghei-infected erythrocytes and saponin released "free" parasites.
2. The determination of Hypoxanthine, Xanthine and Uric Acid in the purine salvage pathway of malaria parasites.
3. First-pass absorption and clearance of the antimalarial drug, Chloroquine, by perfused, whole pig livers.
4. Lipid metabolism in malaria: the identification and quantitation of fatty acids.
5. Biochemical effects of Mefloquine (WR142490) on thyroid function in the rat.
6. Purine uptake in promastigotes of Leishmania braziliensis panamensis.
7. Short interval metabolism of amino acids in promastigotes of Leishmania braziliensis panamensis.
8. Phthalate plasticizers in biological fluid.
9. Drug metabolite studies by mass spectrometry.

1. The uptake of purine bases and nucleosides by the normal rat erythrocytes, Plasmodium berghei-infected erythrocyte and saponin released "free" parasites.

Hansen et al. (1979) have demonstrated the presence of 2 distinct membrane transport loci for the mediated uptake of the purine bases adenine and hypoxanthine and the purine nucleosides adenosine and inosine in normal erythrocytes, Plasmodium berghei infected erythrocytes and saponin released "free parasites". These workers reported two transport loci in the normal erythrocyte, one for purine bases and one for purine nucleosides. However, these uptake systems appear altered upon infection of the erythrocyte and in the saponin released "free" parasite. Adenosine, inosine and hypoxanthine were all mutually competitive inhibitors of one another suggesting a common transport locus for these two purine nucleosides and base. However, Manandhar and Van Dyke (1975) have demonstrated that adenosine may be metabolized at the limiting membrane of the "free" parasite to inosine and then to hypoxanthine, a pivotal purine for transport. If such a mechanism is indeed operating, the observed inhibitions of adenosine, inosine and hypoxanthine upon each other may be due more to metabolism of these substrates than actual competition for the proposed transport loci. To verify the study of Manandhar and Van Dyke (1975) and to clarify our own results, the uptake of ^3H -adenosine was measured in the presence of increasing concentrations of 2-deoxycoformycin, a powerful adenosine deaminase inhibitor. Following a two minute incubation period, a 65% decrease in the uptake velocity of adenosine was demonstrated at a maximal inhibitor concentration of $1\text{ }\mu\text{g/ml}$ (Fig. 1). In addition, aliquots from the post-incubation medium were examined chromatographically to compare the distribution of the ^3H -label from added adenosine in the presence and absence of 2-deoxycoformycin. In the presence of this inhibitor, a significantly greater percentage of the label remained associated with the added adenosine (Table 1). This suggested that the deamination of adenosine had occurred. The initial metabolism by "free" parasites was also examined by using the double label technique of Taube and Berlin (1971). ^{14}C -adenosine labeled only in the base moiety and ^3H -adenosine labeled 70% in the base (8 position) and 30% in the ribose moiety (5' position) were mixed to yield a final ^3H -DPM ratio of 10:1. A cell suspension of "free" parasites was incubated for two minutes with this mixture (adjusted to a final adenosine concentration of 0.01 mM). If the adenosine was directly transported as adenosine, the ^3H -DPM/ ^{14}C -DPM ratio should remain the same. On the other hand, if adenosine was deribosylated, the ^3H -DPM/ ^{14}C -DPM ratio should decrease due to the loss of the ribose moiety and subsequent uptake of the base. Aliquots of the post incubation medium showed a significant decrease in the ^3H -DPM/ ^{14}C -DPM ratio from 10:1 to 5:1. These data suggest that the deamination and deribosylation of adenosine do occur at or on the surface of the limiting membrane of the "free" parasites. Caution must therefore be exercised in suggesting a single transport locus on the membrane of the "free" parasite for adenosine, inosine and hypoxanthine.

Fig 1. The velocity of 0.01 mM ^3H -adenosine uptake (V , $\mu\text{moles/g protein/2 min}$) by "free" parasites as a function of increasing concentrations of 2-deoxycoformycin ($\mu\text{g/ml}$). Each point is the mean of three replicates and the lines were fitted by inspection.

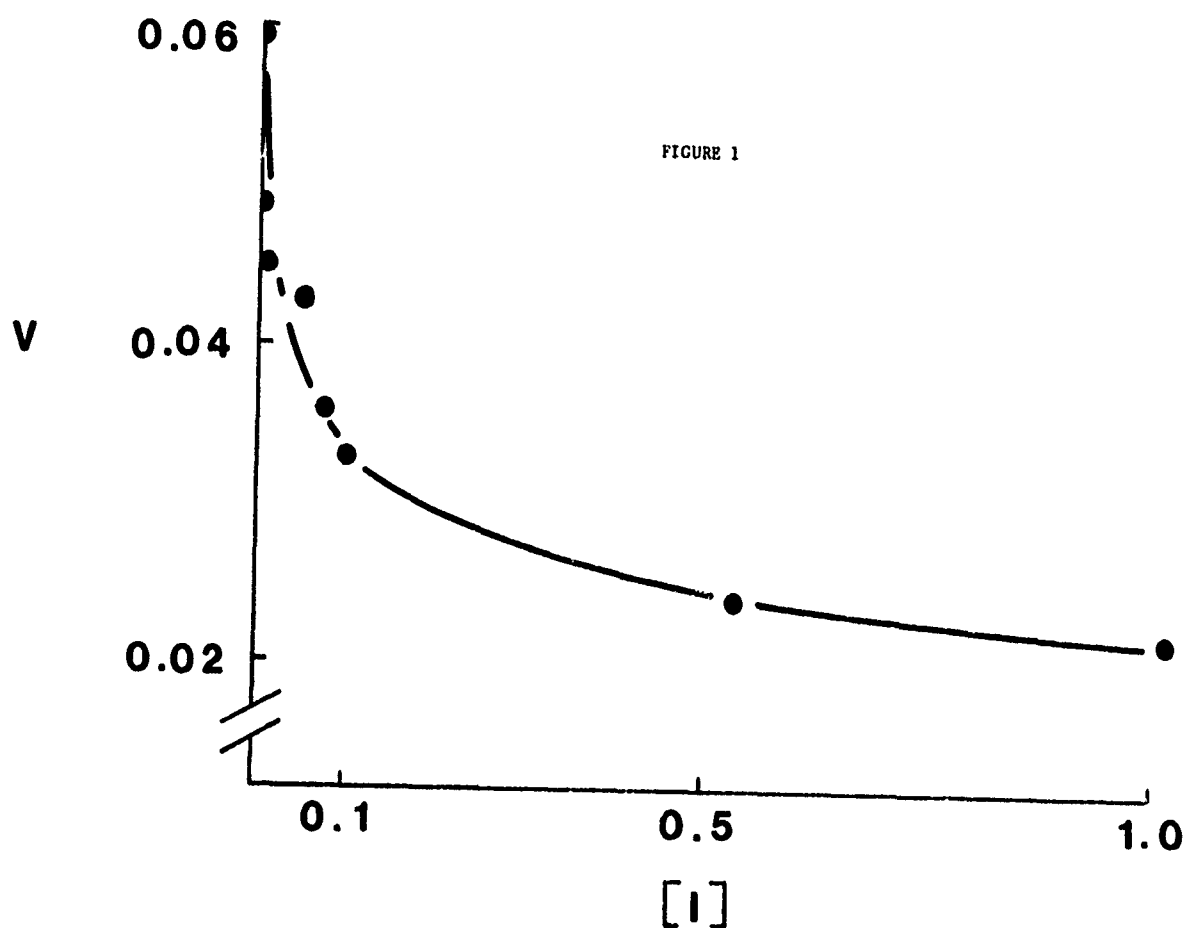


Table 1. The distribution of radioactivity (%) in the post incubation medium of "free parasites" the presence and absence of 2-deoxycoformycin.

	Adenosine %	Hypoxanthine %	Iosine %	Adenine %
Inhibitor	11	51	33	5
2-deoxyco- formycin	62	15	18	5

The cells were incubated at 37 C for 2 min in the presence of 10 μ Ci 3 H-adenosine.

2. The determination of hypoxanthine, xanthine, and uric acid in the pure salvage pathway of malaria parasites.

Numerous methods have been described in the literature for the determination of uric acid in biological fluids, however, few procedures are available which can readily quantify the intermediate metabolites of the purines salvage pathway without employing arduous and time consuming techniques. Because of chemical structural similarities of hypoxanthine, xanthine, and uric acid, interferences between two or more of these compounds have been a major problem in the past.

Recent studies involving the use of high performance liquid chromatography have alleviated many of these difficulties. Using these refinements, we developed a simple and specific method incorporating the use of reverse phase liquid chromatography and paired-ion chromatography for separating these closely related compounds. The method is capable of quantifying these oxypurines at levels as low as 5 nanograms on column. Analysis time is 6 minutes per sample. No pre-treatment other than deproteinization of sample required. Because of the simplicity of the method, its application may directly utilized for various types of basic research projects, as well as for routine clinical determinations in the laboratory.

Our primary interest for developing a method for quantifying the oxypurines was to establish the metabolic profiles of these compounds in plasmodia during the various intra-erythrocytic stages of the parasite. The method is simple and specific for measuring the concentrations of hypoxanthine, xanthine and uric acid.

Nucleotides, nucleosides and free base purines, which are present in our samples did not interfere with the separation. Allopurinol, an analog of hypoxanthine, was also separated by the method.

3. First-Pass Absorption and Clearance of the Antimalarial Drug, Chloroquine, by Perfused, Whole Pig Livers.

Therapeutic regimens of chloroquine (C), an antimalarial drug, causes its concentration in blood and liver. This, in combination with slow rates of metabolism and excretion, emphasize the importance of blood and liver interactions in the effectiveness of C. Interchange of C between these two tissues, its subsequent compartmental distribution, uptake, clearance and metabolism are dictated by the three-dimensional configuration of the organ and its association with the perfusing blood medium. This association was investigated through determinations of C first-pass absorption by the liver, venous outflow profiles, rate of uptake, metabolism and compartmental distribution in isolated, perfused whole pig livers. As described by Baker et al. (Pesticide Biochem. Physiol., vol. 11, 1979), surgically removed livers were placed in a chamber and perfused with recirculating whole blood, during which time ^{51}Cr -labeled red blood cells (vascular reference), methylene blue (extravascular reference) and a therapeutic level of C (test material accessible to all compartments) were added concomitantly as a pulse to the arterial blood. Venous first-pass out flow profiles obtained were characterized by a narrow and high ^{51}Cr peak, a broader and lower methylene blue peak, followed by a still broader and lower C peak. These profiles are consistent with current hypotheses of hepatic transfer rates. Second-pass peak profiles were more shallow and broader than first-pass profiles. First-pass absorption of C by the liver was due to red blood cell uptake, but subsequent transfer of C from red blood cells to liver occurred to a small extent over 60 min. At this time, C was concentrated in red cells > liver > plasma > bile (42:11:1:<1). No metabolites of C were detected by ultraviolet spectrophotometry, but preliminary studies using HPLC have indicated that a small amount of metabolites is present. Throughout the test period, hematological and liver function indices were monitored and maintained at normal levels. The behavior of C in the presence of interacting blood and liver consists of its concentration by red blood cells and to a lesser extent by the liver, its slow metabolism by the liver (hindered by the low metabolic capacity of concentrating red blood cells) and its limited transfer from red blood cell to liver.

4. Lipid Metabolism in Malaria: the identification and quantitation of fatty acids.

Research has continued on the identification and quantitation

of fatty acid by gas chromatography in the plasma and erythrocytes of normal and infected (*P. berghei*) rats, (WRAIR Annual Progress Report '78). The results are shown in Table 2.

In the plasma of non-infected animals, the predominant fatty acid is the C₁₆, with C₁₈, C_{18:1}, and C_{18:2} next most prevalent. In contrast, the plasma from infected animals showed a high concentration of C_{18:1} followed by decreasing amounts of C₁₆, C₁₈, and C_{18:2}. With the exception of the C_{18:1}, the fatty acid levels in the plasma of the infected animals were markedly lower. The C_{18:1} peak could be either Oleic acid or Cis-vaccinic acid which is not resolved under the studied conditions. The erythrocytes from the infected animals had a similar pattern of fatty acid distribution, but with two noticeable exceptions: (1) the C₁₆ was higher in the erythrocytes of the infected animals, and (2) an unknown compound which chromatographed between C₁₄ and C₁₆ was considerably higher in the infected animals. C_{18:3} was found in the plasma of both infected and non-infected animals, but not in the erythrocytes.

Table 2
Fatty Acids Levels (µgm/ml) in Infected and non-infected Rats

Fatty Acid*	Non-infected		Infected	
	Plasma	Erythrocytes	Plasma	Erythrocytes
C ₁₄	4.02	3.99	2.99	3.36
unk	—	5.68	—	14.28
C ₁₆	61.09	23.95	31.22	36.58
C _{16:1}	14.21	—	4.38	2.20
C ₁₈	32.12	20.15	15.86	22.51
C _{18:1}	32.75	12.12	32.15	14.18
C _{18:2}	32.57	11.85	21.56	5.05
C ₂₀	1.46	8.71	1.65	3.98
C _{18:3}	2.06	—	1.43	—
C ₂₂	2.18	5.93	—	—
C _{22:1}	—	10.94	—	—
C _{20:4}	10.91	6.39	2.75	3.48

* Number of carbons in chain, and number of unsaturations.

5. Biochemical Effects of Mefloquine (WR142490) on Thyroid Function in the rat.

Work was continued on the effects of Mefloquine on serum thyroid hormone concentration. All experiments were carried out in male Sprague Dawley rats. Mefloquine was administered orally in doses of 5, 25 and 125 mg/Kg body weight in 35% polypropylene glycol in water. Previous results showed that Mefloquine reduced serum t_4 and T_3 level after 3 to 5 days in high or medium doses (WRAIR Annual Progress Report 1978). The present study examined the short term effect of the drug on serum thyroid hormone levels. Three to 24 hours after drug administration of low and medium doses there was a marked increase in both T_4 and T_3 in the serum. This was followed in the medium dose by a decrease in serum T_4 and T_3 after 24 hours. The high dose suppressed serum T_4 and T_3 as previously reported. The reverse T_3 level in the serum generally paralleled the T_3 levels. The effects of drug during P. berghei infection are being investigated.

6. Purine Uptake in Promastigotes of Leishmania braziliensis panamensis

Promastigotes of most species of Leishmania are apparently not capable of de novo purine synthesis. Therefore purines and their derivatives must be supplied from the host to the parasite. Marr and Berens (1978) demonstrated that radiolabeled formate, glycine and serine were not incorporated into the purine ring. In addition, ^{14}C -glucose was incorporated into both purines and pyrimidines suggesting no de novo synthesis but an active salvage of bases for nucleotide synthesis. Further, Konigk and Rasoul (1978) demonstrated the presence of the purine salvage enzyme adenine deaminase. However, Brazil and M^CCarthy (1979) reported that promastigotes of Leishmania mexicana amazonensis do incorporate the purine precursor glycine but that the pyrimidine precursor orotic acid was not incorporated. This demonstrated reliance of most promastigote species upon purine salvage pathways for the purine ring suggests that the limiting membrane of the promastigote may be of great importance in regulating the absorption of these substrates supplied by the host. The purpose of the present study was to begin the characterization of the purine base and nucleoside transport systems in in vito cultured promastigotes of Leishmania braziliensis panamensis.

The absorptions of 1 mM 3H -adenosine (Fig 2.), 3H -hypoxanthine, 3H -adenine and ^{14}C -inosine were measured as a function of time from 0 to 15 min. Since the labeled purine uptake for 2 min was a linear function of time for all substrates tested, under conditions where the substrate was not limiting (i.e., 1 mM) influx should also be linear under experimental conditions where the substrate is limiting (i.e., less than 1 mM). Therefore, 2 min incubations used throughout our study should provide a reliable estimation of initial influx velocities for these substrates in

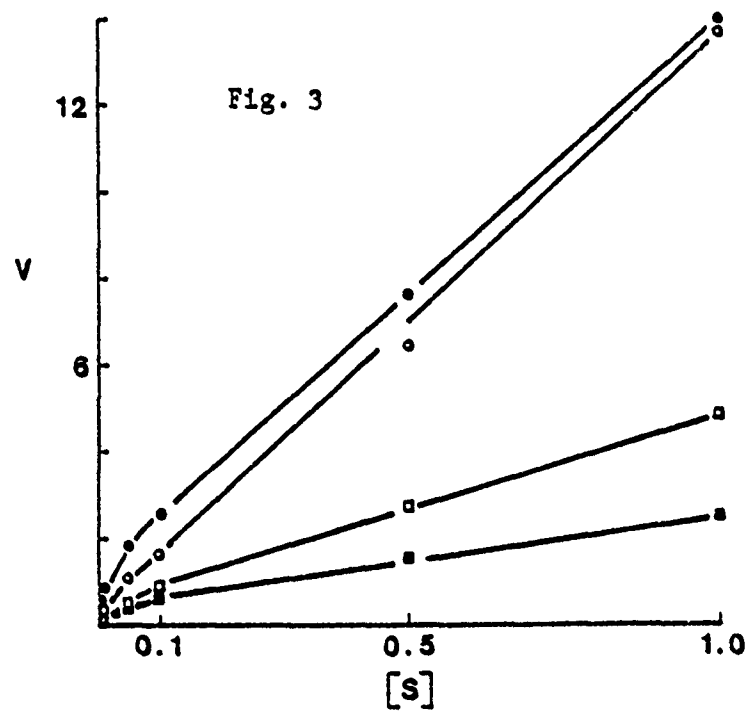
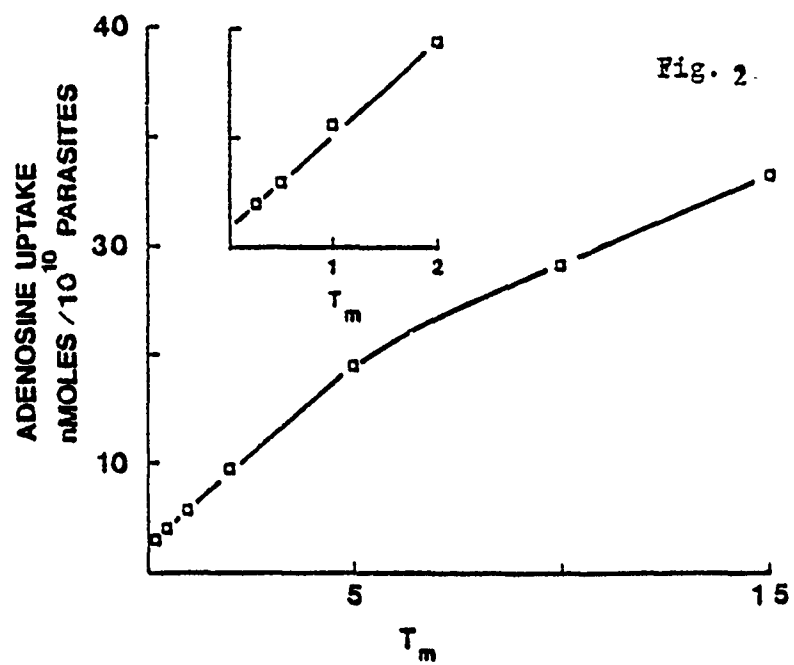
(The uptake velocities of ^3H -adenosine, adenine, hypoxanthine and ^{14}C -inosine (where $V = \text{nmoles uptake}/10^{10} \text{ parasites}/2 \text{ min}$) were measured over a concentration range of 0.001 to 1 mM for 2 min at 27 C (Fig 3). The promastigotes displayed mixed uptake kinetics with regard to these substrates with large diffusion components at high substrate concentrations and mediated components at lower concentrations. The uptake rates of the purine bases adenine and hypoxanthine were significantly greater than the uptake rates of the purine nucleosides adenosine and inosine at all substrate concentrations tested. V_{max} and K_t values for the mediated uptake of these 4 substrates were determined using the method of Lineweaver/Burk and are reported in Table 4.

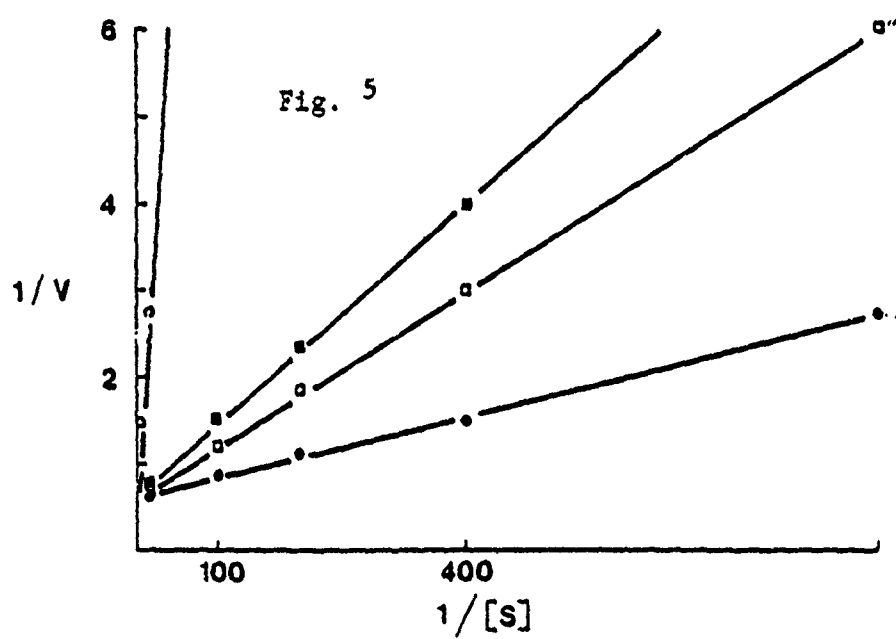
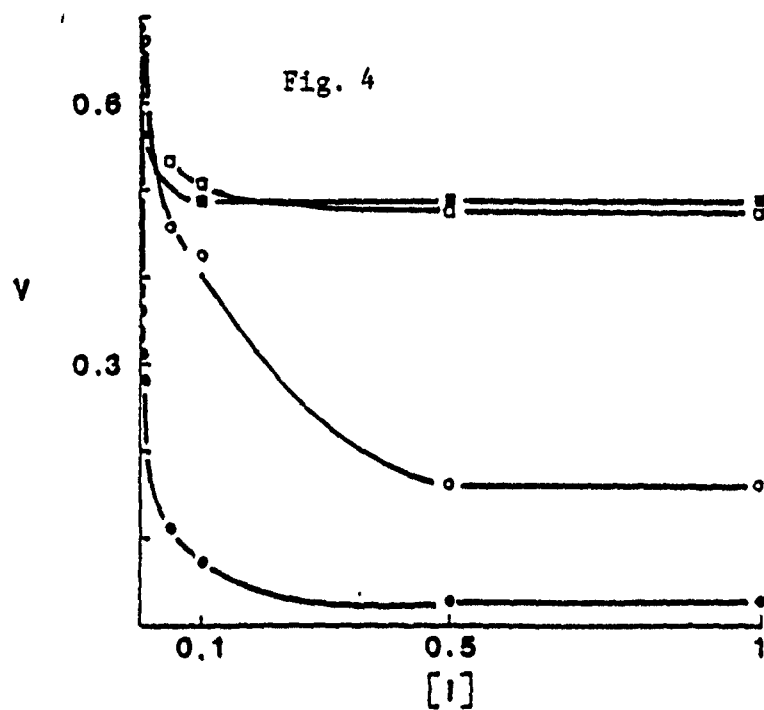
The competitive interactions among these 4 substrates absorbed by promastigotes were measured (Fig 4). The uptake of 10 μM ^3H -hypoxanthine was significantly inhibited by increasing concentrations (0 to 1 mM) of the unlabeled purine bases adenine and hypoxanthine. However in the presence of increasing concentrations of the unlabelled purine nucleosides, adenosine and inosine, inhibition was minimal (i.e., less than 20%). Similar competitive interactions were noted with ^3H -adenosine, ^3H -adenine, and ^{14}C -inosine as the labeled substrates against increasing concentrations of these same purine bases and nucleosides as inhibitors. These data are summarized in Table 5.

The competitive or noncompetitive nature of these inhibitions was also examined (Fig 5). When the reciprocal of the velocity of ^3H -hypoxanthine uptake was measured as a function of the reciprocal of increasing hypoxanthine concentrations in the presence of 1 mM adenine, adenosine, inosine and no inhibitor, the slopes of the plots differed while the Y intercepts did not. The data suggest that these substrates act as competitive inhibitors of ^3H -hypoxanthine uptake. In addition, K_t values from these resulting slopes were also calculated (Table 6). The most significant change in K_t occurred when adenine was present as the inhibitor, increasing some 33 fold when compared to the K_t value for hypoxanthine uptake in the absence of the inhibitor. Because the plot of $1/V$ vs $1/S$ does not differentiate partial from complete inhibition, the uptake of ^3H -hypoxanthine, as previously shown, was measured as a function of increasing concentrations of unlabelled adenosine, adenine and inosine. After correcting these data for diffusion and plotting according to the technique of Dixon (1953) straight lines resulted indicating that the competition is completely competitive for those substrate tested. In this case the reciprocal of the velocity of hypoxanthine uptake is shown as a function of increasing adenine concentration with a linear relationship demonstrated (Fig. 6).

(In summary, these purine bases and nucleosides taken up by Leishmania braziliensis panamensis are absorbed by a combination

of mediated and diffusion systems. In addition the inhibitory interactions among substrates are most likely fully competitive and suggest that 3 membrane transport loci may be functioning as represented by the following model shown in Fig. 7. Locus 1, may bind and transport inosine, locus 2 transports the purine bases adenine and hypoxanthine while locus 3 transports adenosine with hypoxanthine, adenine and inosine binding nonproductively. (Collaborate study with the Dept of Parasitic Diseases, Div of Experimental Therapeutics.)





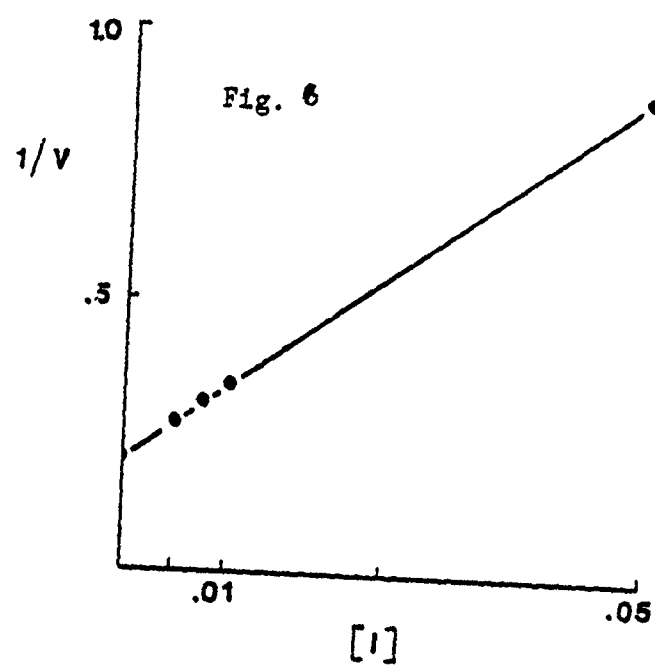
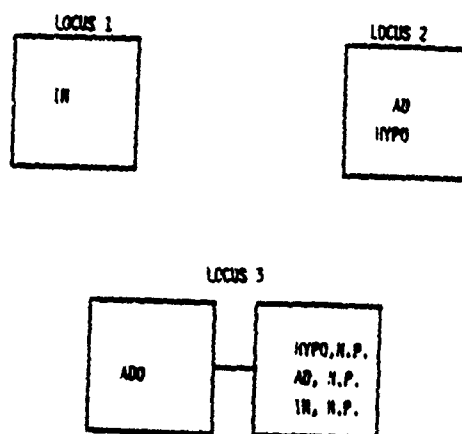


Fig. 7



- Fig. 2. The uptake of (nmoles/ 10^{10} parasites) 1 mM ^3H -adenosine by promastigotes of Leishmania braziliensis panamensis. Each point is the mean of 3 replicates and the lines were fitted by inspection.
- Fig. 3. The velocity (V, nmoles/ 10^{10} parasites/2 min) of ^3H -adenosine () ^{14}C -inosine () ^3H -adenine () and ^3H -hypoxanthine () uptake as a function of increasing substrate concentration (S, mM). Each point is the mean of three replicates and the lines were fitted by inspection.
- Fig. 4. The velocity of 0.01 mM ^3H -hypoxanthine uptake (V as in Fig 2) by promastigotes of Leishmania braziliensis panamensis as a function of increasing concentrations of unlabeled adenosine () inosine (), adenine () and hypoxanthine () as inhibitors (I, mM).
- Fig. 5. The reciprocal of the velocity (V as in Fig 2) of ^3H -hypoxanthine uptake by promastigotes of Leishmania braziliensis panamensis as a function of increasing concentrations of hypoxanthine in the absence of inhibitor () and in the presence of 1 mM adenosine (), 1 mM inosine () and 1 mM adenine (). Each point is the mean of three replicates and the lines were fitted by inspection.
- Fig. 6. The reciprocal of the velocity of uptake (V as in Fig. 2) of 0.01 mM ^3H -hypoxanthine by promastigotes of Leishmania braziliensis panamensis as a function of increasing concentrations of adenine as an inhibitor (I, mM). Each point is the mean of three replicates and the lines were fitted by inspection.
- Fig. 7. A model for the transport of purine bases and nucleosides by promastigotes of Leishmania braziliensis panamensis (AD, adenine; ADO, adenosine; HYPO, hypoxanthine; IN, inosine; N.P., nonproductive binding).

TABLE 3

DISTRIBUTION OF RADIOACTIVITY (%) IN THE
POST-INCUBATION MEDIUM OF PROMASTIGOTES OF
LEISHMANIA BRAZILIENSIS PANAMENSIS

COLD SUBSTRATE	LABELED SUBSTRATE			
	ADENOSINE	HYPOXANTHINE	INOSINE	ADENINE
ADENOSINE	86	4	3	1
HYPOXANTHINE	4	89	5	2
INOSINE	2	3	85	2
ADENINE	12	1	1	92

ALL CELLS WERE INCUBATED AT 27C FOR 2 MIN IN THE PRESENCE OF
10 μ ci OF LABELED SUBSTRATE. THE NUMBERS REFER TO THE PERCENT
OF THE TOTAL RADIOACTIVITY RECOVERED.

TABLE 4

VALUES OF K_t (mM) and V_{max} (NMOL/10¹⁰
PARASITES/2MIN) FOR THE UPTAKE OF LABELED
PURINE BASES AND NUCLEOSIDES (AS DETERMINED
BY LINEWEAVER AND BURK, 1934) BY PROMASTIGOTES
OF LEISHMANIA BRAZILIENSIS PANAMENSIS.

SUBSTRATE	K_t	V_{max}
ADENOSINE	0.006	1.0
HYPOXANTHINE	0.003	1.53
INOSINE	0.008	0.45
ADENINE	0.011	0.5

TABLE 5
THE PERCENTAGE OF INHIBITION OF 0.01 mM ^3H -SUBSTRATES
(CORRECTED FOR DIFFUSION) BY 1mM UNLABELED INHIBITORS
(100:1 INHIBITOR TO SUBSTRATE RATIO) IN PROMASTIGOTES
OF L. BRAZILIENSIS PANAMENSIS.

1mM COLD	0.01mM LABELED SUBSTRATE			
	ADENOSINE	HYPOXANTHINE	INOSINE	ADENINE
ADENOSINE	74	—	—	—
HYPOXANTHINE	46	97	—	54
INOSINE	67	—	94	—
ADENINE	51	53	—	68

PERCENTAGE INHIBITIONS ARE GIVEN WHEN SATURATION LEVELS WERE ATTAINED.
THE NUMBERS REPRESENTS THE % INHIBITIONS OF THE MEDIATED UPTAKE COMPONENT
WITH RESPECT TO THE ABSENCE OF THE INHIBITORY PURINE BASE OR NUCLEOSIDE.

TABLE 6

VALUES OF K_t (mM) AND V_{max} NMOLE/10¹⁰
PARASITES/2MIN FOR THE UPTAKE ³H-HYPOXANTHINE
IN THE ABSENCE AND PRESENCE OF 1mM SUBSTRATE

SUBSTRATE (1mM)	K_t	V_{max}
NO INHIBITOR	0.003	1.50
ADENOSINE	0.005	1.50
INOSINE	0.007	1.50
ADENINE	0.100	1.50

7. Short Interval metabolism of Amino Acids in promastigotes of Leishmania braziliensis panamensis

There is relatively little information with regard to the uptake and utilization of amino acids by promastigote and amastigote forms of the species Leishmania. Using ¹⁴C- labeled substrates and thin layer chromatography, Wagner and Krassner (1976) demonstrated that arginine and onithine were precursors of proline synthesis in in vitro cultures of L. Tarentolae promastigotes. Law and Mukkada (1979) also demonstrated that the same organism also actively transported proline, against a concentration gradient. Further, Camargo et al. (1978) examined the enzymes of ornithine-arginine metabolism of Leishmania and reported the presence of arginase. Numerous nutritioned studies have also demonstrated the amino acid requirements of Leishmania. Steiger and Meshnick (1977) measured quantitative changes of amino acid pools in the external medium of in vitro cultures of Leishmania and found significant decreases in the pools of 13 amino acids and the excretion of 3 others after 4 days. The purpose of the present study was to examine the short interval metabolism of ¹⁴C- labeled amino acids in the promastigote forms of Leishmania braziliensis panamensis.

Promastigotes from in vitro cultures were harvested after a 6 day incubation at 27 C. The cells were washed and placed in a Krebs Ringers Tris Maleate buffer (5 mM glucose) with the following ¹⁴C-substrates: alanine, glutamate, aspartate, glutamine,

glucose, proline, arginine, ornithine, leucine and glycine. Following a 10 minute incubation the cells were washed of exogenous radioactivity and extracted in 70% ethanol overnight. The ethanol extracts were then centrifuged, the supernatant removed and the pellets hydrolyzed in 6 N HCl overnight. Aliquots from the protein hydrolysates were analyzed for total radioactivity by scintillation spectrometry. The ethanol extracts were treated in two ways: (1) 0.1 ml aliquots were analyzed to determine total radioactivity from each sample. (2) 1.9 ml aliquots were analyzed for amino acid content using a Technicon automatic analysis system. Aliquots coming off the ion-exchange column were collected every 3 minutes and analyzed for radioactivity. Radioactive peaks were identified by comparing them to retention times of labeled standards run previously.

The % distribution of ^{14}C from ethanol extracts of those substrates tested and the percent of the total label recovered in the amino acid fraction from the ethanol extracts are reported in Tables 7 and 8. Some label was found in the protein hydrolysates.

When promastigotes were incubated 10 min in the presence of ^{14}C -labeled glutamate, aspartate, glutamine and glucose, > 20% of ^{14}C -label from the ethanol extracts was recovered as alanine (> 50% of the ^{14}C -label of glucose and aspartate were recovered as alanine). The data suggested that when the energy needs of the cells are met (in 5 mM glucose) the alanine free pool increased and may be utilized as an energy store. The promastigotes were also analyzed for the presence of glutamate-pyruvate transaminase, a key enzyme for the conversion of glutamate and pyruvate to alanine. The products of the reversal of this reaction (glutamate and pyruvate) are both actively incorporated into the TCA cycle with an ultimate production of energy. Relatively high levels of both glutamate-pyruvate transaminase and glutamic-oxalacetic transaminase were found in promastigotes of L. braziliensis (Table 9).

When promastigotes were incubated in the presence of ^{14}C -arginine and ^{14}C -ornithine, > 60% the label was found in proline (Table 8). Camargo et. al. (1978) have demonstrated the presence of arginase in L. braziliensis and would explain the presence of ^{14}C label from arginine appearing in ornithine. Presumably proline is then synthesized from ornithine from the ornithine free pool.

Leucine and glycine remained largely unmetabolized during the short interval incubation (Table 8). A listing of proline and alanine precursors are presented in Table 10.

(Collaborative study with Dept. of Parasitic Diseases, Div. Experimental Therapeutics).

Table 7 % Distribution of ^{14}C in *L. braziliensis* amino acids following 10 min incubations in ^{14}C -substrates.

Amino acid	^{14}C substrates (10 μci)				
	Alanine	Glutamate	Aspartate	Glutamine	Glucose
Solvent Front	3.9	8.43	4.35	2.4	31.6
Cysteic Acid	13.6	6.36	17.10	6.9	9.6
Aspartate	1.5	3.56	2.52	3.4	.
Glutamine	0.6	6.83	2.85	17.6	.
Alanine	76.1	29.5	50.46	23.0	53.1
Glutamate	4.3	36.6	11.84	43.0	5.7
Threonine	.	.	1.0	.	.
Glycine	.	1.6	1.0	.	.
Total CPM/ 10^{10} cells in etoh extracts	350,120	279,558	1,022,652	412,380	82,257
% total label in amino acid fraction of the ethanol extract	96.1	91.6	95.6	97.6	68

Table 8. % distribution of ^{14}C in *L. braziliensis* amino acids following 10 min incubations in ^{14}C -substrates.
 ^{14}C substrates (10 μci)

Amino acid	Pro	Arg	Orn	Leu	Gly
Solvent front				1.0	
Cysteic acid urea	.	8.2	.	2.7	0.13
Aspartate	.	0.3	.	.	0.10
Proline	98.3	60.0	99.0	.	.
Alanine	1.7	0.8	.	1.54	.
Ornithine	.	8.0	1.0	.	.
Arginine	.	18.0	.	.	.
Glutamine	.	.	.	22.4	0.10
Glutamate	.	.	.	1.3	.
Leucine	.	.	.	71.0	.
Glycine	98.3
Total CPM/10 ¹⁰ cells in etoh extract	528,444	619,650	62,991	651,753	6,470,766
% total label in amino acid fraction of the ethanol extracts	100	100	100	99	100

Table 9. Activities of Glutamic-Oxalacetic and Glutamate-Pyruvic transaminase in promastigotes of L. braziliensis.

Enzyme	International Units/l (25°C)	
	Promastigote	Human serum
Glutamic-Oxalacetic Transaminase	30.24	5-14
Glutamic-Pyruvic Transaminase	28.32	10-17

Table 10 The formation of ^{14}C alanine and ^{14}C proline by promastigotes of L. braziliensis[†] from ^{14}C labeled precursors.

^{14}C Proline precursors	^{14}C alanine precursors
L- ^{14}C Ornithine	L- ^{14}C Glutamate
L- ^{14}C Arginine	L- ^{14}C Aspartate
	L- ^{14}C Glutamine
	L- ^{14}C Glucose
	*L- ^{14}C Leucine

† The cells were incubated at 27 C for 10 min in KRT buffer with 5mM Glucose

* Trace levels

8. Phthalate plasticizers in biological fluids.

Plasma specimens from patients on kidney dialysis were analyzed for diethyl-hexyl phthalate (DEHP). Samples taken before dialysis and one, two and three hours after dialysis had commenced were extracted for phthalates. The extracts were chromatographed and quantified by gas chromatography to determine the increase in DEHP leached from polyvinyl chloride (PVC) tubing used in the dialysis procedure. Since phthalates were found to be present in the solvents employed in the extractions, prior distillation of these solvents was necessary, although it served only to reduce the amount of DEHP rather than eliminate it completely. Hence, the slight increase of DEHP in plasma for a dialysis period of three hours was of no significance, because the actual concentration of DEHP for this same period did not exceed that of the reagent blank within the limits of variation for the procedure. While the increase in phthalate was consistent, no significance could be derived as the actual amount was too low to be of any consequence. No toxicity was manifested by the patients. Under these circumstances which involved very low concentrations, this was to be expected, since DEHP has comparatively low toxicity, and none of the more toxic analogues were detected during the course of analyses for metabolites. These results are similar to data reported by Vessman and Rietz (11) who concluded that only large volumes of blood stored in polyvinyl chloride containers and subsequently administered to patients could pose a risk of loading the individual with phthalate. Protein fractions, other than lipoprotein, bind very little of the phthalate and hence the phthalates are considered to be no phthalate leached from PVC tubing during periods of dialysis does not increase the possibility of toxic reactions.

This work satisfies the existing protocol, and no further experiments have been planned. Unless or until a new protocol is submitted by our collaborator, Dr. Everett K. Spees, Jr., the task is considered completed.

9. Drug metabolite studies by mass spectrometry.

Efforts to develop simple, routine methodology for terbutaline and metaproterenol have had very little success. Applications of high performance liquid chromatography (HPLC) for this purpose have been quite disappointing. The difficulty lies mainly in the preponderance of lipid material extracted with the extremely small quantity (1 to 10 ng.) of unchanged drug. No matter how sensitive the equipment can be made to respond to terbutaline or metaproterenol, the drugs are masked by the lipid substances to the extent that all indication of terbutaline or metaproterenol are obliterated.

While chemical ionization mass spectrometry has produced the only reliable data thus far, the procedure is cumbersome in that considerable time and skill are required to tune the instrument to the degree of peak performance necessary for optimum sensitivity. Therefore, it will not serve the needs of the clinical laboratory as a routine procedure. Nevertheless, sensitivity is excellent (1 to 3 ng.) and the specificity that can be attained by the gas chromatographmass spectrometer will enable the project pharmacological studies to proceed as intended.

Since most published data on terbutaline have been obtained from labeled radio-isotope studies, no definite information is available as to the exact nature and quantity of metabolites. Contrary to some reports of a sulfate metabolite in the blood, we have, as yet, been unable to confirm such a compound, except in the urine. Only unchanged terbutaline or metaproterenol have been found in the bloods analyzed in our laboratory after administration of therapeutic doses of these drugs. However, some preliminary data on samplings of urine after a 20 mg. dose of metaproterenol indicate that no conjugation is evident in the early phase of drug absorption. Measurable amounts of the unchanged metaproterenol are excreted intact in the urine in lieu of any conjugated drug. Eventually, conjugation of the drug must take place because asthma patients maintained on metaproterenol do have significant concentrations of the conjugated drug in their urine.

A series of experiments are planned that will determine the dose response and derive some data on the metabolism and distribution of metaproterenol and terbutaline. Since many asthma patients are maintained on theophylline as well as metaproterenol, some of the study will focus on the possibility of drug interaction and/or potentiation.

Although a viable procedure for terbutaline and metaproterenol analysis was required to study the pharmacokinetics of these compounds in the treatment of asthma, the effort was motivated by the desire to extend such a procedure to the determination of related catecholamines. This would open new possibilities for the study of nerve response mechanisms in conjunction with chemical warfare agents. Lhuguenot and Maume (12) have reported a similar application to the analysis of catecholamines in rat adrenal glands. Their procedure is essentially the same as our method, and the sensitivities are comparable.

Project: 3M162770A803 DRUG DEVELOPMENT
Work Unit 088 Biochemical Research on Antimalarials

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
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23. (U) To determine the effect of conventional and experimental antimalarials in the treatment, prophylaxis, and transmission of drug-resistant falciparum malaria. To define vector species and bionomics which influence the transmission of malaria and to develop rationales for vector control. To characterize the cellular immune response of patients infected with malaria. To establish strains of human plasmodia in continuous in-vitro culture. To evaluate candidate antimalarial drugs against simian malaria.							
24. (U) Army investigational antimalarial drugs are compared with standard drugs in treatment of drug resistant falciparum malaria in hospitalized human volunteers. Lymphocytes from malaria-infected patients are isolated and their response to malarial antigens characterized. Chemotherapeutic drugs are studied in rhesus monkeys with P. cynomolgi.							
25. (U) 78 10 - 79 09 Mefloquine treatment of falciparum malaria continued to result in radical cure of all patients studied. Mefloquine also cured acute attacks of vivax malaria, although it was not sporonticidal against either infection. Characterization of the response of lymphocytes for malaria-infected patients to non specific mitogen and malaria antigen continued. Isolation of malarial antigen, starting with a technique for production of erythrocyte-free intact parasites, was in progress. Specific cell immune functions are altered in humans naturally infected with malaria and work to determine the interactions of lymphocytes, serum factors and parasite antigen during malaria infection is continuing. The rhesus monkey - P. cynomolgi system was used to test 32 drugs for radical curative effects. Phase II of final definitive testing of WR 225448 was completed. Preliminary studies on the possible use of the cynomolgus monkey (Macaca fascicularis) as an animal model in the malaria drug development project were initiated. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 78 - 30 Sep 79.							

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Project 3M162770A803 DRUG DEVELOPMENT

Work Unit 089 Field studies on drug resistant malaria

Investigators.

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1. Treatment of the Acute Attack of Malaria Caused by Plasmodium falciparum: A Comparison of Mefloquine with Standard Therapy

OBJECTIVE: To determine the effect of several therapeutic regimens upon sexual and asexual parasitemia with P. falciparum in naturally infected humans.

BACKGROUND: Falciparum malaria in Thailand is now considered, for all practical purposes, resistant to chloroquine. As a result, treatment is either with quinine and/or a combination of Sulfadoxine-Pyrimethamine (Fansidar). Other treatment regimens using combinations of tetracycline, quinine and/or sulfamethoxazole-trimethoprim are occasionally employed, but are inferior in clearing parasites.

Quinine continues to be the standard drug given for P. falciparum; its efficacy is well known. A few scattered reports of slow response to quinine do continue to cause concern (1). Fansidar's efficacy in falciparum malaria has been proven, with radical cure rates ranging from 80-90% (2). Apparently there is little difference in cure rates between two or three tablet dose regimens in infected adult Thais. Mefloquine hydrochloride, developed by the US Army Antimalarial Drug Development Program, has been shown to be 94% curative in a single oral dose (3).

METHODS: This study is being carried out as a companion effort to that dealing with the treatment of acute vivax malaria, the results of which are reported elsewhere in this Annual Report. The conditions and methods of patient selection and laboratory studies are described in that report. Patients were randomly assigned to one of the following oral therapeutic regimens:

1. Mefloquine hydrochloride, single dose 1,500 mg.
2. Fansidar, single: a) two tablets, b) three tablets.
3. Quinine, 650 mg. every 8 hrs. for 7 days
4. Quinine, 650 mg. every 8 hrs. for 7 days plus primaquine, 15 mg. for 5 days.

Patients were normally retained in the hospital until clearance of parasitemia and clinical symptoms. They were followed weekly for 28 days, and at their final visit, they were given primaquine 15 mg. daily for 14 days.

Treatment results were evaluated according to WHO criteria originally conceived to evaluate chloroquine resistance and are described in an accompanying report.

RESULTS: One hundred and nine patients have been studied to date. It is apparent from Table 1 that Mefloquine is at least as good as quinine alone and quinine with primaquine when comparing fever clearance times and parasite clearance times. These data suggest however, that Fansidar in the two tablet dose is slower than the other modes of therapy in clearing fever.

In Table 1, treatment failures are defined as failure to clear parasites in seven days and to remain clear for 28 days. In this regard, there were no treatment failures with Mefloquine but Fansidar in both doses and quinine with and without primaquine showed a total of 7 treatment failures. The resistance patterns were equally divided between RI and RII types (Table 2).

Parasitemia detected before day 28 may have been due to reinfection rather than recrudescence as some of these patients returned to malarious regions.

Of the commercially available drugs, quinine continues to be the drug of choice for the treatment of moderate to severe falciparum malaria. The results reported here, show Fansidar, in either the 2 or 3 tablet dose, to be less effective but still an acceptable form of treatment. Previous reports have shown that a combination of 3 days of quinine followed by a single 3 tablet dose of Fansidar, to be 96% curative (3). Mefloquine continues to be the most effective mode of treatment in experimental studies when treating patients with mild to moderate malaria who do not require parenteral medication.

Routine hematological and biochemical parameters have not reflected any ill effects of the different modes of therapy. We have seen no new side effects of mefloquine.

The effects of the therapy regimens on the sexual parasitemias will be addressed elsewhere in this Annual Report.

Table 1. Therapy of the Acute Attack of Falciparum Malaria

Therapy	Number of Patients	Mean Initial Asexual Parasite count/mm ³	Mean Fever Clearance Time in Hours	Mean Parasite Clearance Time in Hours	Number of Treatment Failures*
Mefloquine	25	33,299	60 (N-22)	72	0
Fansidar (2 tabs)	20	22,232	80 (N-16)	71 (N-18)	4
Fansidar (3 tabs)	22	24,729	55 (N-19)	68 (N-19)	4
Quinine	21	22,413	57 (N-21)	69 (N-21)	2
Quinine + primaquine	21	30,415	72 (N-20)	78 (N-20)	2

* Failure to clear parasitemia within 7 days of the initiation of treatment and remain clear for 28 days. Fever and parasite clearance times for RII resistance types were not included in the computation of mean values.

The number of patients shown in parenthesis, varied because some records were incomplete.

Table 2. Results of Treatment of Falciparum Malaria

Regimen	Total	Resistance Patterns				Cure Rate
		RI	RII	RIII	S	
Mefloquine	25				25	100%
Fansidar (2 tabs)	20	2	2		16	80%
Fansidar (3 tabs)	22	2	2		18	81%
Quinine	21	2			19	90%
Quinine + primaquine	21	1	1		19	90%

2. Treatment of the Acute Attack of Malaria Caused by Plasmodium vivax: A Comparison of Mefloquine with Standard Therapy

OBJECTIVE: To determine the effect of several therapeutic regimens upon sexual and asexual parasitemia with P. vivax in naturally infected humans.

BACKGROUND: Vivax malaria appears to be on the increase in Thailand. Standard therapy of P. vivax infections in Thailand consists of 1500 mg of chloroquine administered over the course of three days. Other therapy includes a combination of Sulfadoxine and Pyrimethamine (Fansidar). Fansidar is being used in increasing amounts for the therapy of vivax and falciparum infections in hospitals, clinics, and as self-treatment.

The new antimalarial, Mefloquine hydrochloride, has been shown to be useful in the therapy of the acute attack of vivax malaria (4). It is also useful in P. falciparum infections (2). Primaquine is gametocytocidal against the sexual forms of both P. vivax and P. falciparum in very small doses.

METHODS: This study has been carried out in two malaria - endemic areas in Thailand. The project was initiated at the Phrabuddabat Hospital, in Saraburi Province and later moved to the Phraya Paholpolpayuhasena Hospital, the Kanchanaburi Provincial Hospital. Most recently, the study has moved back to the Phrabuddabat Hospital. Patients were admitted from either the out-patient department of the hospital or the National Malaria Eradication Project detection center. Admission criteria of study subjects were:

1. Males at least 18 years of age.
2. Willingness to volunteer for hospitalization and follow-up. The procedure was carefully explained to the patient, and he was asked to sign a statement of understanding and agreement.
3. Uncomplicated disease of mild to moderate severity.
4. Asexual parasite count between 1,000 and 100,000/cu.mm.
5. Initially, gametocytes had to be present on the initial thick film; however, this requirement was dropped during the study.

Patients were randomly assigned to one of the following therapeutic regimens, all of which were given orally:

1. Mefloquine hydrochloride, single dose, 1,500 mg.
2. Fansidar, single dose: a) two tablets, b) three tablets.
3. Chloroquine 1,500 mg. total dose given over three days.
4. Chloroquine 1,500 mg. as above plus primaquine 15 mg. daily for five days.
5. Pyrimethamine in dosages ranging between 50 and 150 mg. Patients were normally retained in the hospital until clearance of parasitemia and clinical symptoms. They were followed weekly for 28 days, and at their final visit, they were given primaquine 15 mg. daily for 14 days.

Treatment results were evaluated according to the WHO criteria originally conceived to evaluate chloroquine resistance: "S" indicated clearance of asexual parasitemia and maintenance of a negative blood film for 28 days after therapy. "RI" refers to initial clearance of parasitemia followed by recrudescence within 28 days after treatment, "RII" indicates initial reduction in the level of parasitemia, but failure to clear in seven days. "RIII" indicates no reduction in parasitemia following treatment.

RESULTS: One hundred and five patients have been studied to date, Table 2. No new patients were added to the pyrimethamine group. In the five comparable groups, the results are unchanged from those previously reported (6). The fever and parasite clearance times for all groups except Fansidar 2 tablets, were similar. The Fansidar 2 tablet group had significantly prolonged fever clearance times. We again conclude that Fansidar is ineffective treatment for vivax malaria and as such, will not be used for the treatment of vivax malaria in further studies. Mefloquine continues to be very effective in the elimination of parasites and fever.

The routine hematologic and biochemical parameters showed no significant difference between the various treatment groups.

There were no reported treatment failures in the Mefloquine or in either chloroquine group. However, in the Fansidar 2 and 3 tablet groups, treatment failures were noted and found to be of the RI and RII types (Table 4).

The calculated cure rate of 90% for Fansidar, (3 tablets), is based on a small number of patients in that group and may not be borne out in subsequent studies.

Because of its proven safety and efficacy, chloroquine remains the drug of choice for the treatment of vivax malaria. Mefloquine appears to be equally effective; however, the drug is not yet available commercially. Fansidar should not be used in vivax malaria.

Table 3 Therapy of the Acute Attack of Vivax Malaria

Therapy	Number of Patients	Mean Initial Asexual Parasite Count/mm ³	Mean Fever Clearance Time in Hours	Mean Parasite Clearance Time in Hours	Number of Treatment Failures*
Mefloquine	31	6,832	40 (N-21)	50	0
Fansidar (2 tabs)	12	7,478	64 (N-11)	80 (N-8)	4
Fansidar (3 tabs)	11	7,342	49	92 (N-10)	1*
Chloroquine	26	10,760	42 (N-20)	51	0
Chloroquine + primaquine	25	8,516	43 (N-18)	42	0

* Failure to clear parasitemia within 7 days of the initiation of treatment and to remain clear for 28 days. The number of patients shown in parenthesis, varied because some records were incomplete.

Fever and parasite clearance times for RII resistance types were not included in the computation of mean values. Patients without fever were not included in computation of mean fever clearance time.

Table 4 Results of Treatment of Vivax Malaria

Regimen	Total	Resistance Patterns				Cure Rate
		RI	RII	RIII	S	
Mefloquine	31				31	100%
Fansidar (2 tabs)	12		4		8	66%
Fansidar (3 tabs)	11		1		10	90%
Chloroquine	26				26	100%
Chloroquine + primaquine	25				25	100%

3. In Vitro Evaluation of Antimalarial Drugs Against
P. falciparum: A Micro Technique

OBJECTIVE: To monitor chloroquine sensitivity of strains of P. falciparum. To evaluate the described "micro" technique of Rieckman (6).

BACKGROUND: The Department of Medicine has monitored the in vitro susceptibility of P. falciparum to chloroquine in areas where in vivo drug testing has been done.

METHODS: The techniques used are unchanged to those used and reported in the last Annual Report (5).

RESULTS: During the period November 1978 to July 1979, for logistical and technical reasons, no new data were collected. Results to date are in the AFRIMS Annual Report 1977-1978 (5). Since establishment of the field study site in Phrabuddabat Hospital, work on in vitro drug testing has been restarted.

4. Investigation of Possible Quinine and Fansidar
Resistant Cases of Falciparum Malaria in a Refugee Camp

OBJECTIVE: To investigate the reported occurrence of R₃ Quinine and Fansidar resistant cases of P. falciparum malaria in a Cambodian Refugee Camp.

BACKGROUND: During the late rainy season of 1978, it was the impression of the resident physician at the Aranyapratet Refugee Camp (and was agreed to by physicians at other refugee camps) that R₃ level Quinine and Fansidar (sulfadoxine 500 mg and pyrimethamine 25 mg) resistant cases of falciparum malaria were occurring among his patients.

Usual therapy for malaria at the Camp, regardless of type, consisted of three tablets of Fansidar and oral quinine to be taken for six days. If oral medication could not be tolerated, intramuscular Fansidar and intravenous quinine such time as oral medication could be substituted⁷. Both oral and parenteral therapy regimens have been shown to be effective, although parenteral administration is perhaps slightly less satisfactory than the oral regimen^{8, 9}.

The Camp maintained a prophylaxis program consisting of either three tablets of Fansidar given at one time at monthly intervals or one tablet every week for especially vulnerable personnel (approximately 300). These regimens have been shown to be over 85% effective in well-controlled trials^{10,11}.

METHODS: Records on those patients seen prior to arrival of the AFRIMS team who had been treated with the above regimens and subsequently developed a positive malaria smear within one month were reviewed ("old patients").

Malaria slides were prepared on all new cases of possible malaria during a two week period using Giemsa stain (a total of 89 cases) and the corresponding Fields' stain slides, prepared by the Camp's technicians, were examined in 71 of these cases ("new patients"). In addition, slides of ten old patient cases, read as positive for malaria by the Camp technicians were examined. All slides were read independently by three experienced technicians in the Department of Epidemiology, AFRIMS. An examination for 30 minutes was required before a slide was declared negative. In the event of disagreement between the readers (2 cases) the slide was examined by the Chief, Department of Epidemiology.

RESULTS: Thirteen old patient cases were studied. These had occurred over the previous four months; Fansidar and/or quinine achieved less than optimal results in all of them. During July through October 1978, 563 cases of malaria were diagnosed at the Camp (89.7% P. falciparum) with the vast majority (at least 90%) receiving oral therapy on an outpatient basis, but specific quantitation of numbers of patients receiving oral or parenteral therapy was not possible.

Discussion of old patient cases will be divided into responses to the three regimens described above, namely oral therapy, parenteral therapy and chemoprophylaxis (Case reports). With the exception of one case of cerebral malaria, all of these cases eventually resolved on Fansidar and quinine therapy. Where additional drugs were used, they are also noted.

Five patients maintained parasitemias for prolonged periods after oral Fansidar therapy. This period lasted 3,5,6,6 and 22 days in cases 1-5 respectively. The first three of these were hospitalized for the entire period. The fifth case was treated as an outpatient and no smears were obtained between days 1 and 22. One case lasting 6 days occurred during the study period and the patient was admitted, but not for malaria. Camp laboratory technicians reported positive malaria smears on days 2 and 7. Smears taken on days 2 and 3 by our technicians were negative.

In regard to parenteral therapy, five patients did not respond favorably. One case (6) succumbed to cerebral malaria 48 hrs. after admission. Case 7, after apparent recovery from falciparum malaria, required retreatment 18 days after treatment with three vials (mg equivalent to three tablets) of Fansidar IM, intravenous quinine for four days and oral quinine for three additional days. Oral Fansidar and quinine at that time did not prevent convulsions presumably due to cerebral malaria, but the patient recovered on supportive care and IV quinine. Cases 8 and 9 maintained parasitemias 6 and 7 days respectively after initiation of therapy and case 10 represents a mixed vivax and falciparum infection with recrudescence of both types 13 days after parenteral Fansidar.

Cases 11 and 12 represent failures of weekly prophylaxis and case 13 represents failure of the monthly regimen.

Assuming that all of the "old patient" smears had been read correctly, (in only one case do we have information that directly bears on these cases), cases 1-4, 8,9 and 11-13 do not meet WHO criteria for designation of resistance¹². Case 5 may be R₁ resistance or reinfection, but cases 7 and 10 are clearly R₁

(

recrudescences. Case 6 (and possibly case 7) would be termed R₃. The record is not clear at what point in the progression to coma, treatment with Fansidar was initiated, but early failure of therapy was evident. Three chemoprophylaxis failures in approximately 1200 person-months is comparable to that found in the clinical trials¹³ and is probably close to an operational minimum for a prophylaxis program.

Seventy-one Camp prepared and read blood films (Fields' stain), taken from new patients, were available for comparison with Giemsa stained slides prepared and read by AFRIMS technicians. Of these 71 slides, the Camp technicians read 10 as positive (nine P. falciparum and one P. vivax). Of the comparable Giemsa stained slides only two were read as positive. Examination of the Fields' stained slides by Institute technicians resulted in only the same two individuals being diagnosed as malaria (both falciparum). In two cases where the Fields' stained slide was unavailable, but the Giemsa stain was positive, the Camp technician also called the case positive. In one case P. vivax was found in a Giemsa stained slide that was read as negative on the Fields' stain both by the Camp technicians and technicians of the Institute.

Therefore, a 67% false positive rate (8/12) and an approximately 1% false negative rate (1/77, 61 of these Fields' stain confirmed by the Institute) was determined. The one false negative slide was due to staining technique rather than reader error. (In addition, five of the Camp's ten "positive reference" slide sets were read as negative).

CASE REPORTS

Case Histories - Aranyapratet Refugee Camp
(Day 1 will always be date of presentation to hospital)

1. Case 600/21 23 year old male.

Day 1: Smear P. falciparum
Treatment: Fansidar (iii tab)
Quinine (IV x 2 days)

Day 3: Smear: P. falciparum
Treatment: Quinine (IV x 1 day, po x 4 days)

Day 4: Afebrile.

2. Case 2339/19 33 year old male.

The patient worked in the forest for 10 days
prior to presentation.

Day 1: Smear: P. falciparum
Treatment: Fansidar (iii tab)
Quinine (IV x 5 days)

Day 3: Drop in temperature.
Smear: P. falciparum
Treatment: Bactrim (po x 5 days)

Day 4: Smear: P. falciparum

Day 5: Afebrile
Smear: P. falciparum
Treatment: Quinine (po x 3 days)

3. Case 858/21 68 year old.

Day 1: Febrile
Smear: negative
Treatment: Fansidar (iii tab)

Day 6: Febrile
Smear: P. falciparum

4. Case 1895/21 17 year old male.

The patient was admitted for anthrax of the lip and (R) cheek

Day 2: Febrile

Smear: P. falciparum

Treatment: Fansidar (iii tab)
Quinine (IV x 4 days)
Ampicillin
Kanamycin

Day 5,6: Afebrile

Day 7: Febrile

Smear: P. falciparum

5. Case 1410/21 15 year old

Day 1: Smear: P. falciparum

Treatment: Fansidar (iii tab)
Quinine (IV x 2 days)

Day 22: Smear: P. falciparum

Treatment: Fansidar (ii tab)

6. Case 2711/19 41 year old Chinese female. The patient had not left the Camp for three years. She had no past history of malaria.

Day 1: Vertigo and headache progressing to deep coma within 18 hours.

Afebrile

Smear: negative

Treatment: Quinine (IV)
Fansidar (IM)

Day 2: Febrile

Smear: negative

Transfer to Aranyapratet Hospital

Smear: P. falciparum

Day 3: Died (dx: cerebral malaria)

7. Case 1767/21 26 year old male.

Day 1: Hospitalized

Smear: P. falciparum

Treatment: Fansidar (3 vials IM)
Quinine (IV x 4 days)
Quinine (po x 3 days)

Day 11: Discharged

Day 18: Complaint of headache and fever

Smear: P. falciparum

Treatment: Fansidar (ii tab)
Quinine (po)

Day 22: Convulsions requiring hospitalization

Smear: P. falciparum

Treatment: Quinine (IV)
Valium (IV)

8. Case 1278/21 21 year old male.

The patient worked in the forest for one month prior to presentation.

Day 1: Smear: P. falciparum

Treatment: Fansidar (IM)
Quinine (IV x 1 day, po x 1 day,
IV x 2 days, po x 1 day,
IV x 1 day)

Day 4,5: Smear: P. falciparum

Treatment: Procaine penicillin (po x 8 days)

Day 6: Smear: P. falciparum

became afebrile.

Day 7: Smear: Negative

Constitutional symptoms unresolved.

Day 9: Treatment: Bactrim (po x 5 days)

Day 11: Treatment: Quinine (IV x 1 day)

Patient discharged.

9. Case 1218/21 25 year old male.

Day 1: Smear: P. falciparum

Treatment: Fansidar (IM)

Quinine (IV x 4 days, po x 2 days)

Day 3: Smear: P. falciparum

Treatment: Procaine penicillin (IM x 3 days)

Day 5,6,7: Smear: P. falciparum

Day 9: Afebrile

10. Case 656/19 12 year old male.

Day 1: Hepatomegaly and jaundice

Day 3: Smear: P. falciparum and P. vivax

Day 4: Treatment: Fansidar (IM)

Quinine (IV x 6 days)

Tetracycline (po x 11 days)

Smear: Negative

Day 7: Afebrile

Day 11: Jaundice resolved; patient discharged.

Day 17: Readmit: Fever, convulsions.

Smear: P. falciparum and P. vivax

Treatment: Fansidar (IM)

Quinine (IV x 4 days,

po x 5 days)

Chloroquine (450 mg x 4 days)

Prednisolone (20 mg x 6 days)

Day 19: Smear: P. vivax

Day 20: Smear: P. falciparum

Day 21: Afebrile

Day 23: Smear: Negative

11. Case 1752/21 25 year old male.

The patient was on Fansidar prophylaxis (÷ tab q week) for 5 months.

Day 1: Symptoms of chills and nausea

Smear: Negative

Treatment: Fansidar (iii tab)
Quinine (po x 1 day)

Day 3: Smear: P. falciparum
Treatment: Quinine (po x 4 days)

Day 6: Smear: Negative

Note: Patient was never febrile.

12. Case 1107/20 25 year old male.

The patient was on Fansidar prophylaxis for approximately 6 months (i tab q week)

Day 1: Smear: P. falciparum

Treatment: Fansidar (iii tab)
Quinine (IV x 3 days, po
x 5 days)

Day 2: Afebrile within 8 hours.

Day, 5: Smear: Negative
Treatment: Bactrim (po x 5 days)

13. Case 785/21 Male nurse:

The patient was hospitalized and treated for P. falciparum malaria and then was placed on Fansidar prophylaxis (iii tablets at the beginning of the month) for two months.

Day 1 (four days after 2nd prophylactic dose):
febrile and vomiting

Smear: Negative

Day 2: Smear: P. falciparum

5. Evaluation of Experimental Antimalarial Drugs for
Radical Curative Activity in the Rhesus Monkey

OBJECTIVE: To evaluate the radical curative effectiveness of selected experimental drugs in rhesus monkeys (Macaca mulatta) infected with Plasmodium cynomolgi malaria.

BACKGROUND: This is a continuation of studies initiated by this Laboratory in 1974. A chronological report of the methodology and results are available in previous SEATO/AFRIMS Annual Reports (1,2). These studies are conducted in association with the Department of Parasitology, Division of Experimental Therapeutics, Walter Reed Army Institute of Research.

METHODS: Rhesus monkeys were inoculated intravenously with sporozoites produced in Anopheles dirus mosquitoes.

A. dirus mosquitoes were fed on P. cynomolgi infected monkeys. This feeding was conducted during the second or third rise in parasitemia and when both male and female gametocytes were present as evidenced by a blood smear. On post-feeding day 14, the sporozoites were harvested from the salivary glands of the infected mosquitoes and diluted in a saline-normal monkey serum solution (1:1) to a concentration of $5-20 \times 10^5$ sporozoites per ml. Preselected, malaria-negative rhesus monkeys were immediately inoculated intravenously with one ml. of the sporozoite solution.

Each monkey was monitored by blood smears daily, beginning on day 7 post-treatment for the development of a parasitemia. When the parasitemia reached $5-25 \times 10^3$ parasites per cmm, test drugs were administered daily for seven days at a predetermined dosage level, based on a mg. of drug/kg. of body weight. To permit evaluation of drug activity against tissue parasitic forms independently of blood schizonticidal activity, chloroquine phosphate was administered simultaneously with each test drug at 5 mg/kg. body weight/day for seven days.

Following administration of the test drug, malaria parasitemia was monitored by examination of Giemsa stained blood smears daily for twelve days and on Monday, Wednesday and Friday thereafter. Prior to 1 March 1978, monkeys which converted to a negative parasitemia and remained so through post-treatment day 20 were splenectomized and monitored an additional 33 days. Those that remained free of malaria parasites during this period were considered cured. After 1 March 1978, monkeys which converted to a negative parasitemia were monitored for 80 days

post-treatment with no splenectomy. Those remaining negative during this period were considered cured. Those monkeys which either failed to convert to negative parasitemias or which did convert to a negative status initially but subsequently became positive again in under 20 days post-treatment were considered not cured. These monkeys are terminated on the particular drug study when this occurs; however, when their parasitemias reach an acceptable level (approximately 5,000/cmm) they are placed on another, different drug. In this manner, it is possible for one monkey to be used to test several drugs provided they "break" with a parasitemia before post treatment day 20.

RESULTS: A total of 32 experimental drugs were evaluated: results are summarized in Tables 5 & 6. This reduced number of drugs tested over previous years is a direct result of the world-wide shortage of rhesus monkeys available for medical research.

Table 5 Summary of Completed Sporozoite Induced Tests in Rhesus Monkeys

Type of Compound	WRAIR Drug Number	Minimum Curative Dose* (mg/kg/day)
8 - Aminoquinoline	234073	** NC(10.0)
	237808	10.0
	238403	NC(10.0)
	238599	NC(3.16)
	238887	NC(3.16)
	238872	NC(10.0)
	239124	NC(10.0)
	239378	NC(3.16)
	240381	NC(3.16)
	241319	NC(10.0)
	234075	NC(0.1)
Acridines	235660	NC(10.0)
	233602	NC(10.0)
	239373	NC(3.16)
Quinolines	229238	1.0
Miscellaneous	232651	NC(10.0)
	235963	NC(10.0)
	237375	NC(3.16)
	240595	NC(10.0)

* Administered orally with 5.0 mg/kg/day of chloroquine phosphate.

** Not Curative - The compound did not cure at the maximum dose tested or tolerated. The maximum dose is indicated in parentheses.

Table 6 Summary of Drugs Currently Undergoing Sporozoite Induced Tests in Rhesus Monkeys

Type of Compound	WRAIR Drug Number	Dosage Level Currently Being Tested (mg/kg/day)
8 - Aminoquinoline	237807	10.0, 1.0, 3.16, 0.316
	238605	1.0, 0.316, 0.1
	238608	10.0, 3.16, 1.0
	238850	1.0, 3.16
	239372	1.0, 0.316
	241320	1.0
	242047	1.0
	242471	1.0
	242511	1.0, 0.316, 0.1
Quinoline	3863-D-O	1.0
Miscellaneous	230190	1.0
	234737	1.0
	237797	10.0, 1.0, 0.316

6. Drug Tolerance Study of WR 225448 in Rhesus Monkeys

OBJECTIVES:

1. To determine the maximum tolerated dose of selected antimalarial compounds.
2. To characterize the nature of the toxic effects, including a determination of organ systems affected.

BACKGROUND: While certain chemical compounds are known to have excellent schizonticidal activity, they are, at the same time, toxic to the host. The purpose of this study was to determine the toxic dose of WR 225448 and also to determine what organ system(s) were affected by the drug.

METHODS: This study was accomplished in two phases:

1. Secondary Test Phase (Graduated Dosage Study)

The candidate compound was administered orally to each of 2 rhesus monkeys using the method described by Davidson, et al. (1975) (16). This test phase was accomplished in March 1978 and final results are awaiting the histopathological examination of the tissues collected at necropsy.

2. Primary Test Phase (Fixed Dosage Study)

Following completion of the secondary test, a primary test, or fixed dosage study, was initiated in June 1979. Four monkeys were given the maximum tolerated dose as determined in the secondary test phase which for WR 225448 was 10.0 mg/kg. body weight/day. One monkey was included as a control and only the vehicle, methyl cellulose, was administered to it.

Blood was collected once a week from each monkey beginning 14 days prior to the administration of the test drug (WR 225448) and the following laboratory tests performed: RBC, WBC, Differential, Hematocrit, SGOT, BUN, Total Serum Protein, Blood Glucose, and Creatinine.

Additional blood specimens were collected and the above listed laboratory tests performed on three more days during the course of the study as indicated in Table 7.

The dosage of WR 225448 remained 10 mg/kg. body weight throughout the study. Monkeys that died during the study were necropsied immediately and tissues from all organ systems collected for histopathological examination.

RESULTS: Four rhesus monkeys, #G406, G392, G411, and H-4 were given 10 mg/kg. body weight beginning on 18 June 1979. Monkey #G397 served as a control.

Monkey #406 died on 23 June 1979 after receiving five doses of WR 225448. Monkey #G392 died on 24 June 1979 after six doses of the drug. Monkey #G411 died on 27 June 1979, 3 days after the last dose. Monkey #H-4 died on 28 June 1979, 4 days following the last dose of WR 225448. The control monkey, #G397, was euthanized and necropsied on 5 July 1979.

All four test monkeys died before the scheuled completion date of the study, suggesting that the 10 mg/kg. body weight dose exceeds the maximum tolerated dose originally determined by the secondary test phase. Complete necropsies were performed on all the monkeys. Based on gross necropsy observations and laboratory tests performed the liver appears to be the target organ. (See Table 8) Final results of this study are awaiting completion of histopathologic examination of the tissues.

Table 8 Liver Enzyme Levels

MONKEY NUMBER	SGOT/SGPT				
	Day -14	Day -17	Day +1	Day +8	Day +15
G 392	34/25	34/28	46/42	**/	
G 406	34/20	34/28	46/34	**/	
G 411	34/22	40/20	50/30	1460/1800	**/
H-4	20/17	20/17	30/22	800/1340	**/
G 397*	37/25	34/25	46/14	40/28	40/54

* Control

** Died

G 406 - Died Day +5

G 392 - Died Day +6

G 411 - Died Day +9

H-4 - Died Day +10

7. Comparative Study of WR 225448 and Primaquine in the Plasmodium cynomolgi - Rhesus Monkey Radical Curative Model, Phase II.

OBJECTIVES:

1. To compare the efficacy of WR 225448-chloroquine with the radical curative combination of primaquine-chloroquine.
2. To compare the efficacy of WR 225448 alone with the combination WR 225448-chloroquine.
3. To determine whether WR 225448 is efficacious in a single oral dose.

BACKGROUND: In rhesus monkeys infected with sporozoites of P. cynomolgi, primaquine (in combination with chloroquine) is a radical curative drug. In this combination, primaquine cures most infections at a total dose of 3.5 mg. base per kg. body weight, whether that dose is a single dose or divided into 3 or 7 daily doses.⁽⁵⁾

In man, the toxicity of primaquine precludes administration in a single curative dose. Thus, to achieve a radical cure of P. vivax in man, the dose is ordinarily given in divided doses over 14 to 21 days. (in conjunction with a 3-day course of chloroquine).

WR 225448, an experimental 8-aminoquinoline, appeared to be as potent as primaquine as a tissue schizonticide and had exceptionally potent blood schizonticidal activity. The toxicity of WR 225448 in the rhesus is currently being investigated.

Because of the apparent blood schizonticidal activity of WR 225448, the ability to achieve a radical cure of sporozoite induced P. cynomolgi without simultaneous administration of a second drug such as chloroquine was considered a real possibility. Since preliminary testing of WR 225448 indicated that it had a better therapeutic index than primaquine, it was decided to also test its efficacy by a single oral dose.

The dose ranges (Table 9) selected for this study were based on assumptions that the new lot of WR 225448 used in this study would have approximately the same potency as the succinate salt used in preliminary studies and that WR 225448 would cure at the

same total dose whether it was given in a single dose or in seven divided doses.

METHODS: Rhesus monkeys were inoculated intravenously with 5-20 X 10^5 P. cynomolgi sporozoites produced in Anopheles dirus mosquitoes. Each monkey was then assigned, by random selection, to a particular drug-dose regimen. (See Table 9)

Administration of drug in each monkey was initiated on the day after its initial parasitemia reached 5000/cmm. (chloroquine was given beginning on this day in the appropriate groups). Parasitemia was determined in each monkey, by blood smears, three times prior to inoculation, daily from day six post inoculation until three days after parasitemia was suppressed to zero, then every other day through day 40 and twice weekly thereafter. During relapses or recrudescences of parasitemia, counts were made daily.

Monkeys in which parasitemia were cleared by drug was monitored through day 40 at which time they were splenectomized and then monitored for an additional 30 days. If negative at the end of this period they were considered cured. Monkeys in which parasitemia was not cleared by drug were terminated on day 40.

Monkeys in which the parasitemia was cleared by drug but then reappeared before day 40 were treated with chloroquine phosphate, orally, 5 mg/kg. for seven days (whether or not chloroquine was included in the original regimen). If the parasitemia was cleared by the chloroquine, splenectomy was performed 20 days after clearance and the monkey monitored an additional 30 days. Splenectomized monkeys in which parasitemia reappeared were treated with chloroquine phosphate, orally, 5 mg/kg. for 7 days. Those animals in which parasitemia was cleared by this chloroquine treatment were monitored for an additional 50 days.

Results of each drug regimen were categorized as Cure, Relapse, or Recrudescence based on the following definitions:

1. Cure: No reoccurrence of parasitemia following original treatment.
2. Relapse: Failure of the curative drug to clear all tissue parasites. Confirmed by administration of chloroquine to all monkeys with recurrent parasitemia followed by a temporary clearance of the parasitemia.

3. Recrudescence: Failure of the curative drug to clear all blood parasites. Confirmed by administration of chloroquine to all monkeys with recurrent parasitemia followed by a permanent clearance of parasitemia.

RESULTS: Results for each treatment regimen are summarized in Table 10.

A combination of WR 225448 and chloroquine was clearly the most potent regimen. In combination with chloroquine, the minimum curative dose of WR 225448 was 0.875 mg. base/kg. body weight. Administered alone, WR 225448 was definitely curative at 7.0 mg. base/kg. body weight and one out of two monkeys was cured at 3.5 mg. base/kg. body weight. Contrary to Phase I, this study appears to indicate that WR 225448 has slightly more tissue schizonticidal activity in combination with chloroquine than alone.

Primaquine, in combination with chloroquine, cured at 7.0 mg. base/kg. body weight and at 3.5 mg/ base/kg. body weight in one of two monkeys. As was determined in the Phase I study, WR 225448 is at least twice as potent as primaquine as a tissue schizonticide when administered alone in a single oral dose. WR 225448 proved to be at least 4 times as potent a tissue schizonticide as primaquine when administered in combination with chloroquine.

Table 9 Treatment Regimens.

TREATMENT GROUPS							Dosage of Controls mg Base/ Kg Body Weight	
Dosage of WR225448 or Primaquine mg Base/Kg. Body Weight	WR225448*	WR225448* & Chloro- quine**	Prima- quine* & Chloro- quine**	CONTROLS			7.0	
				Prima- quine*	Chloro- quine**	Vehicle**	3.1	
	14.0	2***		2			0	
	7.0	2		2				
	3.5	2		2				
	1.75	2						
	0.875	2	2					
0.4375	2	2						
				2				
					2			
						2		

1. In all cases when Chloroquine is given in conjunction with another drug (WR225448 or Primaquine) the dose of Chloroquine was 3.1 mg.Base/Kg. Body Weight.

2. * Single oral dose
 ** Seven daily oral doses
 *** Number of monkeys/dose

Table 1C Summary of Results.

TREATMENT GROUPS							Dosage of Controls mg. Base/Kg. Body Weight	
mg. Base/Kg. Body Weight Dosage of WR225448 or Primaquine	WR225448*	PRIMAQUINE* & CHLOROQUINE**		CONTROLS		Vehicle**		
		WR225448* & Chloroquine**	Primaquine* & Chloroquine**	Primaquine*	Chloroquine**			
14.0	G400-Recrudes- cence G416-Cure		G409-Cure					
7.0	G426-Cure G429-Cure		G413-Cure G408-Cure G427-Cure					
3.5	G403-Cure G407-Recrudes- cence		G405-Cure G412-Recrudes- cence					
1.75	G415-Recrudes- cence G418-Recrudes- cence							
0.875	G402-Relapse G423-Recrudes- cence	G399-Cure G422-Cure						
0.4375	G406-Relapse G410-Relapse	G430-Relapse G434-Relapse						
			G419-Recrudes- cence G421-Recrudes- cence					
				G411-Relapse G431-Relapse				
						G414-No Effect G415-No Effect		
						0		

1. In all cases when Chloroquine is given in conjunction with another drug (WR225448 or Primaquine) the dose of Chloroquine was 3.1 mg.Base/Kg. Body Weight.

2. * Single oral dose

3. ** Seven daily oral dose

8. Evaluation of Macaca fascicularis as a Laboratory Model for Malaria and Hepatitis Research.

BACKGROUND: The world-wide shortage of rhesus monkeys (Macaca mulatta) resulting from the moratorium on the export of this species by the Indian Government has spawned the search for alternate animal species to conduct research in certain areas. The malaria drug development program is among those areas. Indeed, a large portion of the VII Congress of the International Promatological Society held in Bangalore, India, 8-12 January 1979 was devoted to finding alternative subhuman primate species to use in medical research. Other papers, describing the breeding and raising of rhesus by various laboratories around the world, were given. Ironically, it was the consensus of the delegates that destruction of the habitat was the major cause of the declining population of the wild rhesus and that the few (relatively speaking) monkeys used for medical research was of only minor significance in the problem.

As a result of these developments two projects were initiated during the year. The first was an experiment in breeding the cynomolgus monkey (Macaca fascicularis) in our Laboratory. The second was using purchased cynomolgus monkeys in pilot studies in the areas of malaria and hepatitis research.

RESULTS: On 30 November 1978, two breeding groups of cynomolgus monkeys, Macaca fascicularis (Crab eating Macaque), were received. One breeding group consisted of one male and nine females and the other breeding group contained one male and seven females. To date a total of 15 offspring have been born from both groups. See Table 11 and Table 12. The January and February and March offspring (7) have been weaned and are adjusting very well to their surroundings.

On 20 March 1979, ten additional cynomolgus monkeys were purchased. Five of the monkeys were laboratory-reared and five were wild caught. Two of the wild caught (#14, #6) died on 6 April 79 and 15 April 1979 respectively. Cause of death was enteritis and dehydration. Four of the remaining eight monkeys were sent to the Virology Department and placed on Hepatitis A Studies. (#7, #8, #20, #21). Results of these studies are reported elsewhere in this report. Subsequently, cynomolgus #7 died as a result of pneumonia, the diagnosis being based on gross necropsy lesions. Definitive diagnosis is awaiting histopathological examination of the tissues.

The other four cynomolgus monkeys (#4, #10, #9, #13) are being utilized in anticipation of their eventual use in the anti-malaria drug testing program. Initially, the study called for a determination of whether or not Macaca fascicularis (Cynomolgus monkey) could be infected with Plasmodium cynomolgi. Cynomolgus monkeys #4 and #10 were inoculated with 5×10^5 sporozoites on 5 June 1979. The two monkeys developed parasitemias at 9 and 10 days post inoculation respectively. Based on the experience gained from the rhesus monkey model, a decision was made to feed mosquitoes on the second peak in the parasitemia. The parasitemias that developed initially were far lower than that seen in the rhesus model. (3,500 to 12,000 as compared to 200,000 to over 300,000). Likewise, the second peak in the parasitemia was of such limited magnitude and of such short duration that the chance to feed mosquitoes was missed. Consequently, when the mosquitoes were fed, they did not become infected.

Cynomolgus #9 (wild caught) and #13 (laboratory reared) were then infected in the same manner as the rhesus monkeys and in this trial, mosquitoes were fed on both the first and second parasitemic peaks. In this second trial we were able to infect the mosquitoes.

Subsequent trials using the cynomolgus monkey model will have to await a decision to purchase additional monkeys. This work will be continued if the results are promising enough to warrant such further investigation and investment of time, money and laboratory space.

Table 11 Summary of births by month of cynomolgus monkeys

1979 Month	Number born
January	2
February	3
March	2
April	1
May	0
June	0
July	5
August	2
Total	15 (13 ♂, 2 ♀)

Table 12 Summary of cause of deaths within the cynomolgus monkey breeding colony

Cause of Death	Number
Stillborn	2
Parasitism*	1

* Overwhelming infestation of Strongyloides sp.

9. Partial Characterization of Mitogenesis Inhibiting
Factor in Malaria Serum

OBJECTIVE: To further characterize the properties of the mitogenesis inhibiting substance(s) in serum from patients infected with malaria.

BACKGROUND: Recently Wells, et al. (5) demonstrated that sera from patients with falciparum or vivax malaria significantly suppressed the blastogenic response of normal lymphocytes simultaneously stimulated with selected plant mitogens. Significant suppression was seen in the cellular response of lymphocytes stimulated with phytohemagglutinin (PHA) and Concanavalin A (Con A), but not in cultures stimulated with Pokeweed mitogen (PWM). Later experiments using autologous or allogenic responder lymphocytes showed similar results (17) although some cultures stimulated with PWM did show various decreases of suppression.

We are continuing to investigate the inhibiting substance and the possible regulatory role it may have in the host's response to malaria infection. Preliminary studies are underway to determine if both acute and convalescent serum contain the inhibiting factor, if inhibiting serum must be added simultaneously with the mitogen for inhibition to be seen, and if the level of inhibition is altered when different concentrations of inhibiting sera are substituted in the culture media.

We will later begin physiochemical studies to determine the inhibiting substances' molecular weight, if it is dialyzable and/or temperature sensitive, and if its' action is directed toward both B cells and T cells.

METHODS: The mitogenesis inhibition assays are performed as previously described (5,18) with modifications in the methodology for those experiments in which different dilutions of serum are added, or in experiments where inhibiting serum is added after mitogen is added to the specific culture wells.

Acute serum is obtained just prior to patients receiving chemotherapy, while convalescent serum is obtained 14 and 28 days later. The respective samples are frozen at -20°C until the complete set can be tested in a single assay against identical responder cells.

Additional physiochemical and target cell specificity studies will be performed using modifications of standard methods (18,19,20).

RESULTS: Table 13 shows the effect various dilutions of inhibiting sera have on the response of normal lymphocytes to mitogen. The highest SI is seen when normal serum alone, or 15% normal serum plus

5% patients serum, is added to the culture media.

When the percentage of inhibiting serum is increased, a decrease in stimulation index (SI) is seen. These results, including the suppression of PWM induced mitogenesis, are consistent with the recent findings of Wells, et al. (2). The most important finding, however, is that the percentage of inhibiting serum can be reduced to 10% thus conserving serum for additional studies. Additional experiments are underway to confirm these results.

Table 14 shows the effect of adding mitogenesis inhibiting sera to mitogen cultures at different times. Although only PHA was used to induce mitogenesis in this experiment, the results indicate that inhibiting serum must be added at the same time as the mitogen to bring about the highest level of suppression. Additional experiments will be necessary to see if inhibiting serum added at times prior to the addition of mitogen results in a higher level of suppression than in those culture receiving inhibiting serum and mitogen simultaneously.

Two inhibition experiments comparing convalescent serum with acute serum have been performed to date. The results are inconsistent and may tend to indicate that the presence of inhibiting substance in convalescent serum may be related to the chemotherapy the patient receives (6) (reported elsewhere in this Annual Report). This is a preliminary report.

Table 13 Effect Dilution of Inhibiting Sera Has on Mitogenic Response of Normal Lymphocytes.

Mitogen	20% NS ¹ + 0% P ²	15% NS + 5% P	10% NS + 10% P	5% NS + 15% P	0% NS + 20% P
PHA	40.0 ³	43.32	21.6	22.18	31.8
CON A	141.92	132.11	68.70	58.10	101.50
PWM	128.70	127.82	58.91	37.97	56.87

¹NS = Normal human serum ²P = Patient (P79-006 PV) serum ³ = Stimulation index

Table 14 Effect of Adding Mitogenesis Inhibiting Sera at Different Time to Mitogen Cultures.

Mitogen	30% NS ¹ (control)		10% NS + 20% P ² (day 0)		10% NS + 20% P (day 3)		10% NS + 20% P (day 5)	
	CPM	SI ³	CPM	SI	CPM	SI	CPM	SI
PHA	3105.7 338.25	9.2	7106.0 1502.25	4.7	3221.25 452.0	7.1	3810.5 371.25	10.3

¹NS = Normal serum ²P = Patient serum (73 Pf) ³SI = Stimulation Index

10. Nature of Malaria Cold-Reactive Anti-Lymphocytotoxin Antibody

OBJECTIVE: To determine the target cell population and the chemical nature of lymphocytotoxic antibodies in sera from patients infected with malaria.

BACKGROUND: Initial studies in this Laboratory have demonstrated the presence of anti-lymphocytotoxin antibodies in the sera of patients infected with falciparum or vivax malaria (5). These antibodies have anti-lymphocytotoxic activity at 15°C and are effective in destroying allogenic, as well as, autologous lymphocytes. Although the in vivo relevance of these antibodies is unclear, recent interest has focused on the relationship of these antilymphocytotoxic antibodies and the decreased Tcell numbers found in the peripheral blood of patients infected with malaria (21). Our continued investigation in this area is presently concerned with determining the antibody class of the lymphocytotoxin (IgG, IgM, IgA) and the lymphocyte population that the lymphocytotoxins are directed against with the aim of understanding the in vivo relevance of anti-lymphocytotoxic antibodies.

METHODS: Human peripheral blood mononuclear cells will be obtained from freshly drawn blood by fractionation over Ficoll-Hypaque gradients according to the method of Boyum (22). Isolation of human lymphocyte subpopulations will be carried out using current modifications (28, 24) of previously described methods (6). Cytotoxin assay will be performed using the modification of Terasaki's methodology (5) previously described. The nature of the antilymphocytotoxin antibodies will be investigated using a standard absorption technique and double antibody radioimmunoassay (23).

RESULTS: The column cell separation assays have been set up and are presently being standardized; consequently no information is presently available on the target cell specificity of the lymphocytotoxins.

11. Fansidar and Human Lymphocyte Immune Response to Plant Lectins

OBJECTIVE: To determine the effect of Fansidar on the human immune response to selected mitogens.

BACKGROUND: Fansidar is presently used widely in Thailand as an anti-malarial drug by the National Malaria Eradication Project, the Military, and for self-treatment. Recently, questions have arisen concerning the possible adverse effects on the hematopoietic system that long-term prophylactic use of the drug may have (25,26).

Recently, a mitogenesis inhibition assay in our Laboratory showed atypical results when pooled convalescent sera from patients with falciparum malaria who were treated with Fansidar was added to normal lymphocytes. Since mitogen stimulation has been used as an indicator of general cellular immune responsiveness, the in vitro inhibition seen with pooled patient's sera could indicate a drug-induced suppression of

(1) the immune response of individual recovering from malaria infection, and/or,

(2) general immune competence of lymphocytes from uninfected individuals.

Both components of Fansidar, pyrimethamine and sulfadoxine, have been shown to adversely effect hematopoiesis in humans when the drugs were taken daily for extended periods (25,26,27). Thus, it is possible that Fansidar, taken routinely for prolonged prophylactic purposes may likewise adversely effect the individual's immune competence.

We are presently using the mitogen inhibition assay to:

(1) confirm that convalescent sera from patients treated with Fansidar (two tablet regimen) inhibit the mitogen stimulation of normal lymphocytes.

(2) determine if chemoprophylaxis with Fansidar can alter the mitogen response of lymphocytes in individual receiving a single three tablet regimen or if mitogenesis inhibiting activity is present in sera from individuals on long term (20 weeks) chemoprophylaxis (two tablets every 2 weeks).

METHODS: "Serum regulatory factors" and "lymphocytes test populations" will be tested using a standard mitogen stimulation assay (5). Lymphocytes will be isolated from blood using the method of Boyum (28). Serum regulatory factors will be examined by adding media containing 20% (v/v) test sera to cultures containing either normal, autologous, or allogenic lymphocytes, then performing the mitogen stimulation assay described above. Control sera will be obtained from patients treated with quinine. Stimulation index (SI) will be calculated as described (25) and used to measure the non-specific stimulation of lymphocytes as well as the control and test sera's effect on isotope incorporation by normal, mitogen stimulated lymphocytes.

RESULTS: Table 15 shows the effect that pooled patients' sera, collected before and after Fansidar treatment, had on the level of isotope incorporation in mitogen stimulated normal lymphocyte cultures. Acute patients' sera collected before treatment, from both of the groups eventually treated with either quinine or Fansidar, inhibits mitogenesis by normal lymphocytes. This is in agreement with the findings of Wells, et al. (25) in which serum from acutely ill malaria patients (P.f. & P.v.) added to normal lymphocyte cultures prior to mitogen stimulation inhibits mitogenesis.

When pooled convalescent sera is added to lymphocyte cultures, sera from patients treated with quinine has no mitogenesis inhibitory effect. The SI of cells treated with 28 day sera from quinine treated patients is not significantly different from that of cells receiving media supplement with sera from pooled, uninfected, untreated individuals. However, sera from patients treated with Fansidar still had high mitogenesis inhibiting activity 28 days after treatment to all 3 mitogens investigated. Further studies may help to determine if the inhibition is due solely to Fansidar treatment or if the continued inhibition is due to the predisposing malaria infection in combination with Fansidar treatment.

Another experiment designed to see if Fansidar chemoprophylaxis (three tablet therapeutic regimen) altered an individuals' lymphocyte response to mitogen was recently completed. Lymphocytes were tested from individuals before beginning Fansidar prophylaxis and 14 and 28 days later. Each lymphocyte population was tested in a mitogen inhibition assay on the day of collection with normal, autologous patient, and allogenic patient serum. No consistent, significant differences were seen in any of the tests. However, a larger number of individuals will have to be examined to assess the role of Fansidar and drug-induced suppression of human lymphocytes.

We are presently using serum from individuals who have been on Fansidar prophylactic for up to 20 weeks in an effect to determine if prolonged use of prophylactic doses of Fansidar can lead to the appearance of mitogenesis inhibiting activity.

Testing of the sera from the prophylactic study is in progress.

Table 15 Pooled Malaria Patient Serum Effect on Normal Human Lymphocyte Response to Mitogen

Mitogen	Normal Serum	Quinine			Fansidar		
		Day 0 ¹	Day 14	Day 28	Day 0	Day 14	Day 28
PHA	156.43 ²	57.84	94.51	107.97	34.24	40.49	27.40
Con A	166.94	56.33	156.53	136.92	24.72	42.35	70.95
PWM	116.06	42.50	114.95	112.05	4.36	7.87	15.67

1. Pre treatment serum.
2. Stimulation index when pooled sera (average 5 patients) is added to wells (20% v/v).

12. Cryogenic Preservation of Malaria Lymphocytes

OBJECTIVE: To develop the capability for freezing and long-term storage of lymphocytes isolated from the blood of individuals with malaria.

BACKGROUND: Numerous immunological studies have been performed to investigate the humoral response of humans to malaria infection (29,30) while relatively few studies have investigated the role lymphocyte subpopulations may have in either the protection or recovery of individuals from naturally acquired malaria infections.

A major reason for this difference is that the cells must be processed and used quickly, unless facilities are available for controlled freezing. Serum can be processed and stored with a minimum of time and equipment.

For the last several years this Laboratory has been engaged in cellular immune studies using lymphocytes from patients with naturally acquired malaria. Although progress has been made, two characteristics of the epidemiology of the disease have hampered previous studies.

First, because the majority of malaria cases are found in rural areas, collection teams are limited in the number of blood samples that they can obtain, screen, process, and transport each morning. They must allocate enough time to ensure that the processed samples are returned to our laboratory, by late afternoon, for use in assays. Any patients who arrive at the field treatment site after a designated "cut off" time are not bled for cell studies.

Secondly, we are restricted by the seasonal occurrence of the disease. The maximum number of cellular assays are performed during the "peak" season; however, few malaria cellular assay can be performed during the rest of the year.

A controlled cell freezing capability will remove some of the limitations mentioned above and will enable us to:

1. Set up an "intermediate" laboratory facility closer to the blood collection sites where blood can be processed and cells frozen for temporary storage. This will decrease transportation time and will allow technicians to collect blood from volunteers throughout the day. Once the blood is collected it can be transported a short distant and processed and stored at the "intermediate" lab before being transported back to AFRIMS.

2. Collect, freeze, and store patient lymphocytes for use during

the months when the incidence of malaria is lower, so that cellular research experiments can continue throughout the year.

3. Begin sequential in vitro studies of the cellular immunocompetence of malaria patients. These studies previously have been limited by day-to-day test variability, making interpretation of results difficult. Several authors have advocated the use of frozen lymphocytes in these in vitro assays to reduce day-to-day test variation, thereby enabling a sequential set of lymphocytes from a patient to be thawed and tested on the same day (31,32).

METHODS: Cryogenic equipment will be set-up and standardized at AFRIMS before being moved to an acceptable "intermediate" facility.

The Biological Freezing System (Union Carbide Corp. Linde Division, N.Y., N.Y.) to be used includes:

1. A BF-4 rate controller
2. A BF-4-1 freezing chamber
3. A model LS-160 liquid nitrogen container
4. An Electronik One-Eleven Single Pen Strip Chart Recorder (Honeywell International, Fort Washington, Pa.)

Ficoll-Hypaque will be used for the separation of lymphocytes from peripheral blood (33). A standard method of freezing live lymphocytes will be followed (34) and cell samples will be stored in a mechanical freezer at a constant -70°C .

RESULTS: Due to long procurement and delivery times, all of the components for the system were not received until recently. In addition the BF-4 rate controller was found after extensive testing to be defective and had to be returned to the Manufacturer for replacement. We are presently awaiting the replacement rate controller. Because of the above we have no results to date.

13. Gametocytocidal and Sporontocidal Studies of Experimental Antimalarial Therapeutic Regimens

OBJECTIVE: To determine the gametocytocidal and sporontocidal effects of experimental antimalarial therapeutic regimens on the malaria parasites in Anopheles dirus and An. maculatus fed on test malaria patients.

BACKGROUND: One very important aspect of evaluating antimalarial therapeutic regimens is to determine their effect on the sexual cycle of the malaria parasite. This evaluation primarily involves screening the blood of malaria patients for gametocytes over a series of days post-treatment. However, gametocytes are often present at levels too low for detection using standard blood film screening techniques. Another method to determine the presence of healthy gametocytes is to allow known vector mosquitoes to feed on the patient and to monitor, by dissection, the development of parasites (if any) in the mosquitoes. The development of "normal" appearing oocysts, subsequently coupled with heavy infections of sporozoites in the salivary glands is good evidence against gametocytocidal or sporontocidal activity by the test antimalarial regimen.

Gametocytocidal studies have been carried out at this Laboratory over the past several years in conjunction with the U.S. Army's Drug Development Program (1, 2, 3). Data from these studies indicate a definite increase or enhancement of gamogamy and subsequent sporogony with the use of Fansidar treatments (2, 4).

METHODS: During this period studies were carried out at the Phraya Paholpolpayuhasena Hospital, Kanchanaburi Hospital, and the Phra Phutthabat Hospital, Sara Buri Province. The usual conditions for admission of patients to AFRIMS therapeutic trials were observed. Patients with P. falciparum or vivax were assigned to therapy groups using one of the following 5 treatment regimens: Mefloquine hydrochloride (1500 mg-single dose), Fansidar (2 tablets), Fansidar (3 tablets), Quinine (650 mg.q. 8 hours for 7 days) and Quinine (650 mg. q. 8 hours for 7 days) plus Primaquine (15 mg/day x 5 days).

Fifty Anopheles dirus (Bangkok Strain) and 50 An. maculatus (IMR Strain) were fed on patients on the day of admission (Day 0) before treatment and on day 1. Followup feeds using these 2 species occurred on days 7, 14 and 21, if the patient still exhibited parasites on blood smears on those days. Mosquitoes were dissected on days 7 and 14 after feeding. Guts and glands were examined for oocysts and sporozoites, and oocyst indices and sporozoite densities were determined.

RESULTS: There were 66 malaria patients admitted into the test program during the year, and on which mosquitoes were fed. However, since the 1976-1977 Annual Report period (1), criteria for the admission of patients have changed and no longer require the presence of gametocytes on the initial thick smear (day 0). Entomological repercussions of this change resulted in a low rate of parasite positive mosquito feeds. Otherwise, the data accrued for the Mefloquine hydrochloride and Fansidar in 2 tablet or 3 tablet doses are essentially unchanged from previous Annual Reports (1, 2, 3). Data for the 2 remaining therapies indicate Quinine (650 mg.q. 8 hrs for 7 days) has little (if any) gametocytocidal activity, while Quinine (650 mg.q. 8 hrs for 7 days) plus Primaquine (15 mg/day x 5 days) has good gametocytocidal activity.

These studies will be terminated in the near future and pending the arrival of new investigational antimalarial drugs.

Project 3M162770A803 DRUG DEVELOPMENT

Work Unit 089 Field studies on drug resistant malaria

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(U) Leishmaniasis; (U) Drug Development; (U) Biology; (U) Chemistry; (U) Toxicology							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Provide individual paragraphs identified by number. Provide text of each with Security Classification Code)							
<p>23. (U) To find new drugs with chemoprophylactic or chemotherapeutic activity against the cutaneous and visceral forms of leishmaniasis, which poses a serious hazard to military personnel operating in most of the tropical and subtropical regions of the world. The only drugs currently available are antimonials, which are hazardous and frequently ineffective.</p> <p>24. (U) Selected chemical compounds will be tested for antileishmanial activity (1) <u>in vitro</u>, (2) in laboratory rodents, (3) in subhuman primates or canines in a series of leishmania models which have been developed or modified by this laboratory.</p> <p>25. (U) 78 10-79 09. A drug screening system based upon inhibition of the incorporation of radiolabelled metabolites by promastigotes or amastigotes of <u>Leishmania braziliensis panamanensis</u> in culture is being developed. Methods for mass cultivation of leishmania were extended to additional strains in support of biochemical studies, taxonomic studies, and drug development studies. Pentostam in a cream applied topically was effective in causing partial regression of cutaneous lesions of <u>L. b. panamanensis</u> in <u>Myristomys</u>. Chloroquine administered IM in a prophylactic regimen delayed or prevented development of cutaneous lesions after promastigote-induced <u>L. mexicana</u> infection in <u>Myristomys</u> and also exhibited activity against promastigotes, but not amastigotes, in culture. Further studies with liposome-encapsulated antimonial drugs demonstrated potential prophylactic application. Improved sampling and culture techniques developed in this laboratory are being employed in the diagnosis of leishmaniasis, in follow-up of patients and in research. For Technical Report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.</p>							

Project 3M162770A803 DRUG DEVELOPMENT

Work Unit 092 Chemotherapy and Chemoprophylaxis of Leishmaniasis

Investigators:

Principal: MAJ Larry D. Hendricks
CPT Lawrence K. Lightner
CPT George E. Childs
Associate: LT CDR Erich Stafford
Brian Hansen, Ph.D.

1. Description

Leishmaniasis is a parasitic infection of the reticuloendothelial system and is endemic in most of the tropical and subtropical areas of the world. Military personnel operating in Latin America, Asia, Africa and the Near East face a serious hazard of disability, disfigurement and death by the cutaneous and visceral forms of this disease. The only drugs currently available for therapy are antimonials, which are hazardous and frequently ineffective.

The Leishmania Section develops and uses laboratory models for evaluation and development of antileishmanial drugs, monitors contract research efforts, furnishes laboratory support for clinical studies and patient care, and collaborates with investigators in other departments, institutions and organizations in research studies of this disease.

2. Progress

a. In vitro Investigations

(1) Axenic amastigotes

A method for evaluating drugs for activity against host-cell-free amastigotes of Leishmania braziliensis panamanensis in vitro was described in the 1978 Annual Report.

As with many in vitro systems, standardization of the axenic amastigote drug screen has been difficult. The undefined portion of the media used in this system, i.e., 30% v/v fetal bovine serum (FBS), appears to be the most variable factor involved. In addition, most major biological supply companies have recently published advisory letters warning that a critical shortage of fetal bovine serum will occur during the next 18 months. Therefore, our major efforts have been toward replace-

ment of FBS in the growth medium and elucidation of the growth factor associated with it.

We have attempted to replace FBS in the medium with tripeptides, heme, a combination of amino acids or neonatal bovine sera. These experiments have not disclosed any substitute for FBS. Encouraging results have been obtained from evaluation of various growth factor(s) isolated by Drs. Shiigi and Mishell, Department of Bacteriology and Immunology, University of California, Berkeley. They were able to isolate two species of cryophilic bacteria from various lots of "sterile" fetal bovine sera. Supernatant fractions from cultures of these bacteria, identified as "gliding bacterium one" (BG₁) and "two" (BG₂), have been provided to us for testing in leishmanial cultivation. Preliminary investigations with these materials indicate that BG₂ may provide the growth factors found in Mishell-Dutton-approved lots of FBS (see Figure 1). Efforts to further purify and isolate specific factors from these supernatant fractions are underway. These materials will be forwarded from Berkeley to our laboratories for testing as they become available.

(2) Promastigotes

In attempts to develop other in vitro drug screening systems, the incorporation of several labeled metabolic precursors including ¹⁴C-proline, ¹⁴C-leucine and ³H-thymidine by promastigotes of L. braziliensis panamanensis in a 96-well microtiter-plate system is being investigated. While these investigations are still in an early stage, it is interesting to note that many drugs used to treat leishmaniasis in man appeared to have little or no effect on incorporation of labeled precursors by the promastigote stage of the parasite, while drugs such as ethidium bromide (WR 141,377) appeared to have a marked effect (Figure 2). Further investigation of this system using both promastigotes and amastigotes are planned. In vitro studies of leishmanial metabolism currently being conducted by Dr. Brian Hansen, Division of Biochemistry, should provide additional insight as to labeled substances that could be used advantageously in this system.

(3) Mass Cultivation

Methods for the mass cultivation of promastigotes of strains of human leishmania have been successfully extended to several additional strains. Promastigotes were cultured in 100 ml Schneider's Medium with 30% fetal bovine serum in series of roller flasks. Media made in the laboratory have been successfully used as substitutes for commercially prepared media with considerable savings in cost. Approximately one ml of packed cells may be harvested from one liter of medium. Mass-cultivated

promastigotes are being used to support biochemical studies, in vitro antileishmanial drug screening experiments, and taxonomic investigations.

b. Mystromys albicaudatus (African white tailed rat) Model of Leishmaniasis

(1) Leishmania braziliensis panamanensis

The evaluation of L. b. panamanensis infections in M. albicaudatus as an animal model for cutaneous leishmaniasis has continued.

Infections of Mystromys with L. b. panamanensis have been used for testing candidate therapeutic agents mixed with hydrophilic emulsion base (HEB) cream for topical treatment of cutaneous leishmaniasis. In one experiment compositions of pentostam in HEB and silver sulfadiazine in HEB were tested. There were 4 animals in each experimental group and 1 control animal. All animals had well-developed cutaneous lesions prior to application of the cream. The medicated cream was applied twice a day for six weeks. Changes in sizes of the lesions of the treated animals in this experiment are shown in Figure 3. The lesion on the control animal did not decrease in size during the experiment (data not shown). Pentostam in HEB cream was similarly tested in a second experiment (see Figure 4). In both experiments, lesions of the pentostam/cream-treated animals decreased in size markedly throughout the study period, while lesions of control animals did not.

Preliminary studies to compare the activities of glucantime and WR 6026 in the Mystromys-L. b. panamanensis system were completed. Glucantime, administered at a dosage of 1200 mg/kg IM for five days, caused a significant reduction in the size of established cutaneous lesions of infected Mystromys. WR 6026 administered at 13 mg/kg IM for five days also caused a reduction in lesion size. Interpretation of results was difficult, however, because animals injected IM with WR 6026 developed local necrosis of tissue at the injection site.

(2) Leishmania mexicana

Data from previous investigations have shown that M. albicaudatus was susceptible to infection with L. mexicana. It was also demonstrated that when the antimony compound, glucantime, was given at a dosage of 100 mg/kg/day IM for 10 days, commencing one-half to two hours prior to infection, the development of cutaneous lesions was delayed at least seven weeks when

the inoculum of promastigotes was 5×10^6 organisms. This information was incorporated into a research proposal submitted to the Uniformed Services University of the Health Sciences by LT CDR Erich Stafford and MAJ Larry Hendricks. This study entitled "Testing for Leishmanial Prophylactic Drugs" is being conducted collaboratively with the USUHS.

Initial experiments have established parameters of activity for the reference compound glucantime and have demonstrated that WR 6026 given either orally or IM is prophylactically less effective than glucantime as per the criteria established for this test.

Current experiments with primaquine, 15 and 30 mg/kg/day IM for 10 days and chloroquine at 30 mg/kg/day IM for 10 days have provided interesting and unexpected results. At seven weeks post-infection, animals treated prophylactically with primaquine have started to develop cutaneous lesions; however, animals treated with chloroquine have not displayed any clinical evidence of cutaneous lesions. Additional investigations of chloroquine as a potential leishmanial prophylactic will be pursued at the conclusion of this series of experiments.

c. USAMRDC Contracted Leishmanial Projects

The Leishmania Section, Division of Experimental Therapeutics, has been designated by the USAMRDC as monitor and technical advisor for a group of U.S. Army contractors conducting research on leishmaniasis. The research includes the following contracts:

(1) Dr. Bruce E. Beacham, Department of Dermatology, University of Virginia Medical Center, Charlottesville, VA, entitled "Investigation of cross immunity between *Leishmania tropica* (Jericho) and *Leishmania braziliensis* in experimentally infected *Myomys albicaudatus*."

(2) Dr. Robert Herman, Bureau of Biological Research, Rutgers College, Piscataway, New Jersey, entitled "Functional role of humoral antibodies in leishmaniasis in laboratory animals."

(3) Dr. William L. Hanson, Department of Parasitology, The University of Georgia, Athens, GA, entitled "Chemotherapy of leishmaniasis."

(4) Dr. Wallace Peters, Department of Medical Protozoology, London School of Hygiene & Tropical Medicine, London,

England, entitled "Chemotherapy of leishmaniasis."

(5) Dr. Uri Zehavi, The Center for the Study of Infectious and Tropical Diseases, the Hebrew University, Hadassah Medical School, Jerusalem, Israel, entitled "Target oriented drugs against leishmania and trypanosoma."

d. Liposome-encapsulated Drugs

Investigations of the antileishmanial activity of compounds encapsulated in liposomes have continued. Antimonial drugs and certain non-antimonial drugs have had increased activity when administered in this configuration. The selection of the most efficacious lipids for preparation of liposomes and the evaluation of extended shelf-life of preparations have been investigated. The use of liposome-encapsulated antimony preparations administered in a prophylactic regimen has also shown promise, indicating that the half-life of the antimony in the host is extended. A long-term treatment regimen of liposome-encapsulated antimony is also currently being investigated.

It is planned that future experimentation will include the evaluation of liposome-encapsulated compounds against cutaneous leishmaniasis and further evaluation of efficacy of "promising" preparations against visceral leishmaniasis in other mammalian systems.

e. Cryobank Project

The collection of reference strains and new isolates of Leishmania has continued. The current collection consists of 340 specimens including 41 new strains and 50 new stabilates which were added this year. Thirteen of these new stabilates were isolated from patients seen in our laboratory during FY 79. Nine were U.S. Army personnel. Acquired stabilates of particular interest are Uta, a species causing mucocutaneous leishmaniasis in Peru, and new isolates of both visceral and cutaneous leishmaniasis from Kenya that have natural resistance to antimony. Requests from seven laboratories for stabilates from the Leishmania cryobank reference collection have been received this year. In response to these requests, 56 stabilates have been forwarded.

f. Diagnostic Laboratory Services

As a result of a leishmanial EPICON request from Ft. Bragg and a resultant FDA-approved IND for the treatment of Army patients with modified regimens of Pentostam, this laboratory has assumed a major diagnostic mission.

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This year 50 military patients with suspected leishmaniasis have been seen in our laboratories. This group included new suspect patients; patients undergoing antimony therapy requiring evaluation; and patients requiring three-month, six-month, and one-year follow-up after antimony therapy; and a group of 17 West Point cadets that attended Jungle Warfare Training in Panama and had persistent dermal lesions. Of these 50 patients, 8 were confirmed as new cases of leishmaniasis, 15 were previously treated patients, (two of which were positive at 3 and 6 months post-treatment) and two were patients that were undergoing therapy at the end of the last fiscal year.

The Department recently has been involved in collaboration with another EPICON mission involving a Battalion from Ft. Bragg that had returned from Panama. Twenty-nine soldiers were diagnosed as having become infected with toxoplasmosis while participating in jungle training. The isolation of oocysts from the involved watering point, the demonstration of rodent infections from the oocysts via serological methods and the final isolation of oocysts after feeding of infected rodents to felines will include consultation and collaborative efforts by this section.

g. Collaborative Support of Special Foreign Activities

This section has actively participated in the leishmaniasis research efforts of the WRAIR laboratory in Kenya. This has included exchange of materials, isolation and characterization of isolates and consulting visits.

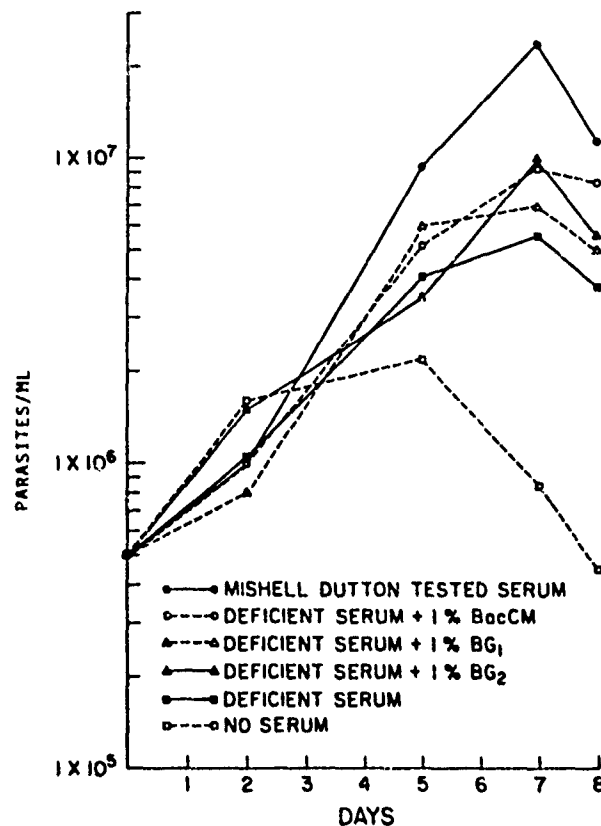


Figure 1. Growth of amastigotes *in vitro* in media containing factors from fetal bovine serum.

Mishell-Dutton Test Serum: fetal bovine serum approved by Mishell-Dutton Test

Deficient Serum: fetal bovine serum which failed Mishell-Dutton Test

BacCM: Bacterial Conditioned Medium: medium remaining after bacteria have been removed from culture.

BG₁: "gliding bacterium one": fraction of a culture of one bacterium found in "sterile" fetal bovine serum.

BG₂: "gliding bacterium two": fraction of a culture of a second bacterium found in "sterile" fetal bovine serum

L-6

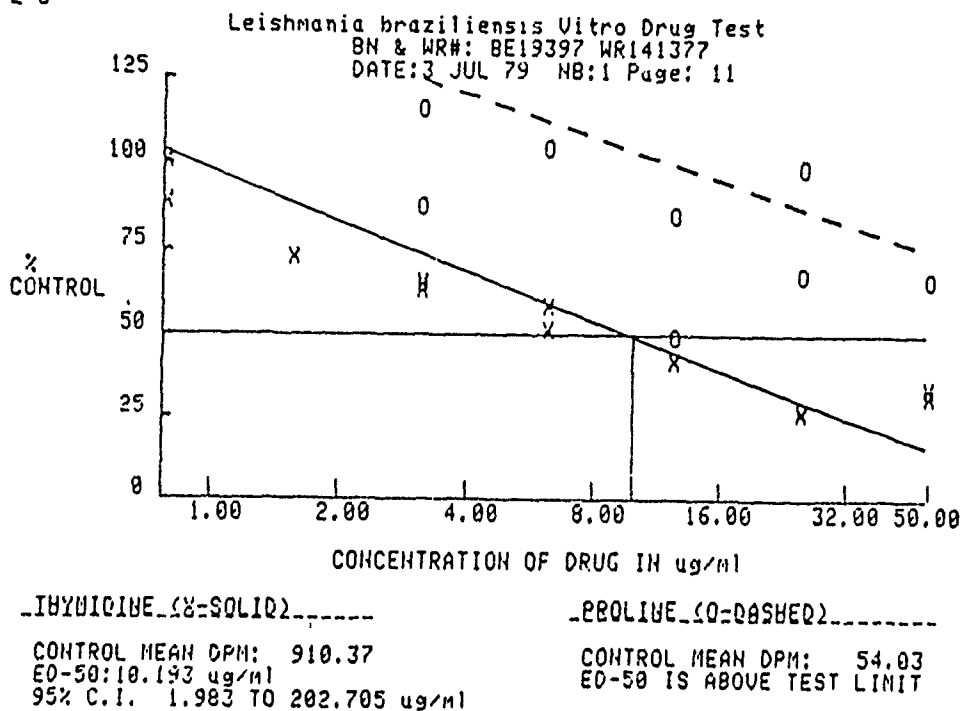


Figure 2. The effect of ethidium bromide (VR 141,377) on the incorporation of ^3H -thymidine and ^{14}C -proline by promastigotes of *L. braziliensis panamanensis* during cultivation in vitro. The figure is a reproduction of the computer-generated display of the analysis of experimental data. Promastigotes were incubated for four hours at 25° in medium containing ^3H -thymidine and ^{14}C -proline. The radioactivity incorporated by the promastigotes in the presence of drug was compared to that incorporated in the absence of drug ("Control") and expressed as percentage of control (% control).

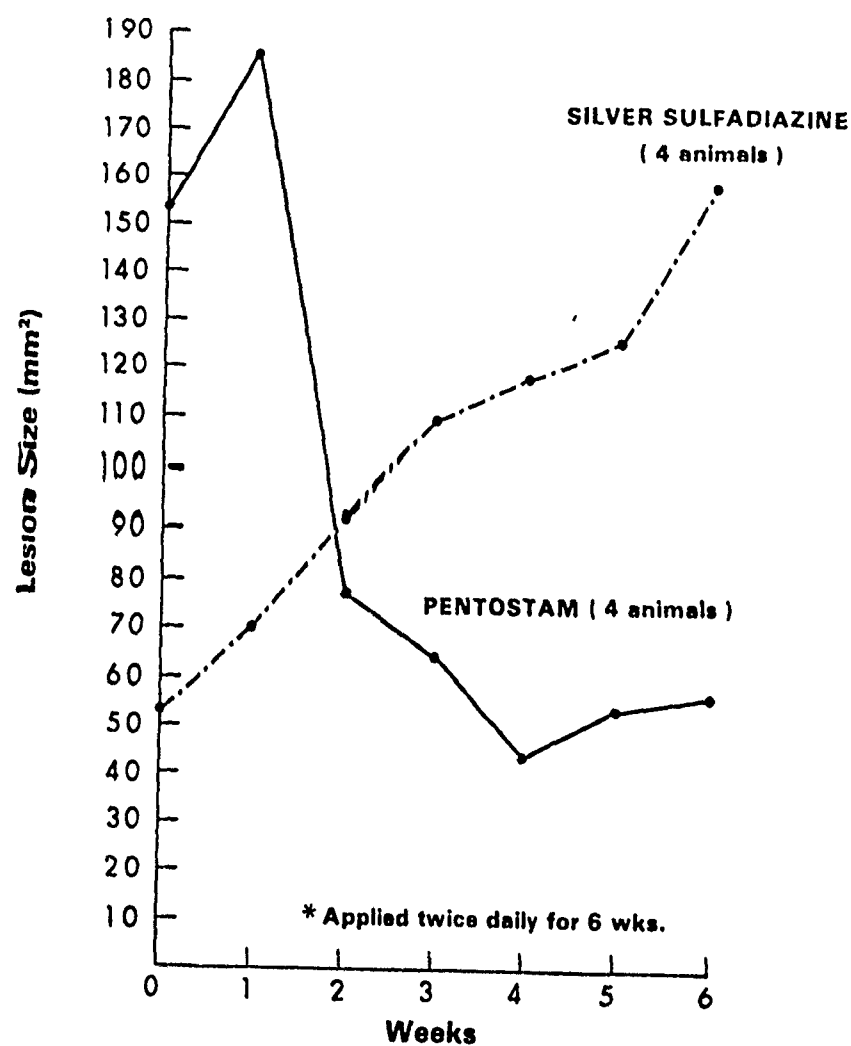


Figure 3. Treatment of Leishmania braziliensis in Mystromys albicaudatus by Application of Pentostam and Silver Sulfadiazine Creams*

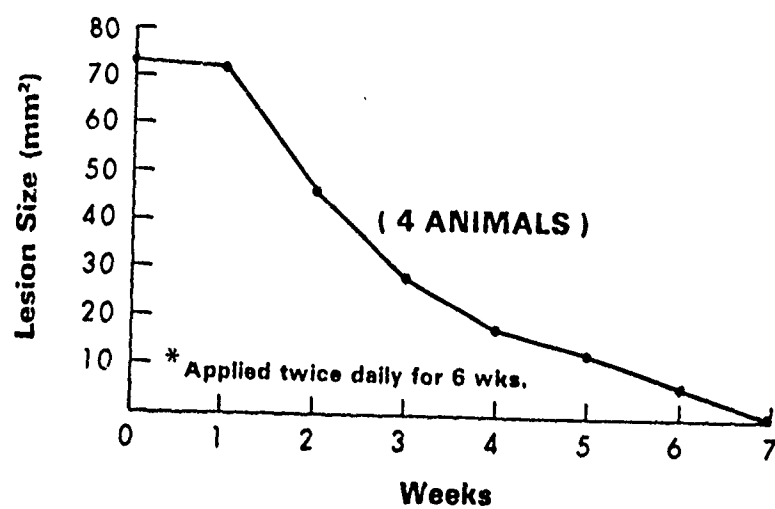


Figure 4. Treatment of Leishmania braziliensis in Myiostomys albicaudatus by Application of Pentostam Cream*

Project 3M162770A803 DRUG DEVELOPMENT

Work Unit 092 Chemotherapy and Chemoprophylaxis of Leishmaniasis

Publications:

a. Papers Presented:

1. Hendricks, L.D. and Childs, G.E.: In vitro cultivation of leishmanial parasites. Presented at the Workshop on In Vitro Cultivation of Pathogens of Tropical Diseases, ILRAD, Nairobi, Kenya, 4-9 Feb 1979.

b. Published:

1. Wilson, H.R., Diekmann, B.S. and Childs, G.E.: Leishmania braziliensis and Leishmania mexicana: Experimental cutaneous infections in golden hamsters. Exp. Parasitol. 47: 270-283, 1979.

2. Childs, G.E., McRoberts, M.L. and Moussa, M.A.: Systems for the in vitro large-scale propagation of New World Leishmania. Ann. Trop. Med. Parasitol. 73(4): 395-396, 1979.

3. Childs, G.E., Foster, K.A. and McRoberts, M.S.: Insect culture media for cultivation of New World Leishmania. Int. J. Parasitol. 8: 255-258, 1978.

4. Hendricks, L.D., Ernst, J.V., Courtney, C.H., and Speer, C.A.: Hammondia pardalis sp. n. (Protozia: Elmeriidae) from the ocelot, Felis pardalis, and experimental infections of other felines. J. Protozool. 26(1): 39-43.

5. Ewert, A., Corredor, A., Lightner, L. and D'Alessandro, A.: Onchocerciasis in Colombia: Follow-up study after 12 years. Am. J. Trop. Med. Hyg. 28(3): 486-490, 1979.

c. In Press:

1. Kinnamon, K.E., Steck, E.A., Loizeaux, P.S., Hendricks, L.D., Chapman, W.L., Jr. and Hanson, W.L.: Leishmaniasis: Military significance and new hope for treatment. Milit. Med. (in press).

2. Cahill, K.M. and Hendricks, L.D.: Leishmaniasis, to be Chapter 60 in An Update on the Zoonoses: 1978. Iowa State University Press (in press).

3. White, S., Hendricks, L.D. and Chulay, J.: Cutaneous leishmaniasis: A case report. Rifampin treatment failure. Published as Letter to the Editor, Arch. Dermatol. (in press).

4. Hendricks, L.D. and Wright, N.: Diagnosis of cutaneous leishmaniasis by in vitro cultivation of saline aspirates in Schneider's drosophila medium. To be published: Am. J. Trop. Med. Hyg. 28(6): 962-964, 1979.

5. Hendricks, L.D. and Childs, G.E.: In vitro cultivation of leishmanial parasites. WHO Symposium (in press).

6. Lightner, L.K., Ewert, A., Corredor, A., and Sabogal, E.: A parasitologic survey for Mansonella ozzardi in the Comisaria del Vaupes, Colombia. To be published: Am. J. Trop. Med. Hyg. 29(1): xxxx, 1980.

7. Hockmeyer, W.T., Kager, P.A., Rees, P.H. and Hendricks, L.D.: The culture of Leishmania donovani in Schneider's insect medium; its value in the diagnosis and management of patients with visceral leishmaniasis. Trans. Roy. Soc. Trop. Med. Hyg. (submitted for publication).

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Project 3E162771A804
MILITARY PSYCHIATRY

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1- AGENCY ACCESSION#	2- DATE OF SUMMARY	REPORT CONTROL: 311 MOL	
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3- DATE: 78 10 01	4- KIND OF SUMMARY: D. Change	5- SUMMARY SCTY: U	6- WORK SECURITY: U	7- REGRADING: NA	8- DISP INSTEAD: NL	9- SPECIFIC DATA- CONTRACTOR ACCESS: <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10- LEVEL OF SUM: A. WORK UNIT
11- NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY	62771A	3E162771A804		00	041		
B. CONTRIBUTING							
C. CONTRIBUTING	CARDS 114F						
11- TITLE (precede with Security Classification Code)							
(U) Behavioral Variables in Autonomic Function and Disease in Military Personnel							
12- SCIENTIFIC AND TECHNOLOGICAL AREAS							
013400 Psychology 012900 Physiology 016200 Stress Physiology 02500 Clinical Medicine							
13- START DATE		14- ESTIMATED COMPLETION DATE		15- FUNDING AGENCY		16- PERFORMANCE METHOD	
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17- CONTRACT/GRANT				18- RESOURCES ESTIMATE		19- PROFESSIONAL WAR YRS	
A. DATES/EFFECTIVE: N/A				PRECEDING		B. FUNDS (in thousands)	
B. NUMBER:				FISCAL YEAR		3	
C. TYPE:				CURRENT		124	
D. KIND OF AWARD:				80		205	
E. AMOUNT:				3			
F. CUM. AMT.							
20- RESPONSIBLE JOG ORGANIZATION				21- PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research Washington, DC 20012				NAME: Walter Reed Army Institute of Research Division of Neuropsychiatry Washington, DC 20012			
ADDRESS:				PRINCIPAL INVESTIGATOR (precede with S. Academic Institution)			
RESPONSIBLE INDIVIDUAL				NAME: Hursh, S.R., CPT (P)			
NAME: Russell, P.K., COL				TELEPHONE: (202) 576-2483			
TELEPHONE: (202) 576-3551				SOCIAL SECURITY ACCOUNT NUMBER:			
22- GENERAL USE				ASSOCIATE INVESTIGATOR			
Foreign Intelligence Not Considered				NAME: Faden, A.I., MAJ			
				NAME: Cuthbert, B.N., CPT			
23- KEYWORDS (precede each with Security Classification Code)							
(U) Physiology; (U) Emotions; (U) Stress; (U) Autonomic Function; (U) Military Psychiatry; (U) Conditioning							
24- TECHNICAL OBJECTIVE, 25- APPROACH, 26- PROGRESS (precede individual paragraphs identified by number. Precede text of each with Security Classification Code)							
23. (U) This is a multidisciplinary effort addressing the development and use of laboratory models to define and describe the organ system responses and disease states caused by stressors in the military environment.							
24. (U) The techniques of operant and respondent conditioning will be employed in the production of models of both phasic and chronic psychological and emotional stress. Cardiovascular and gastrointestinal function will be monitored by electronic transducers and chronic indwelling catheters and fluid samples will be assessed for hematological and hormonal effects. Electrophysiological measurements of central and autonomic responsiveness will provide both a more accurate interpretation of similar data collected in studies with human volunteers and a source of hypotheses relevant to preventive and therapeutic intervention for cardiovascular and gastrointestinal disorders in military personnel.							
25. (U) 78 10 - 79 09 Activities included: Verification that low doses of naloxone reverse the hypotension of endotoxin and hypovolemic shock in rats and dogs with significant improvement in survival. Discovery that naloxone reverses the hypotension of spinal shock and trauma in rats and cats. Derived evidence that naloxone effects may be mediated by action on an endogenous opiate receptor. Development of computer based labs to study environmental modulation of both cardiovascular and gastrointestinal functioning. Supported collaborative efforts to study "jet lag", pulmonary neuropathy, and autonomic dysfunction. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.							

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Project 3E/62771A804 MILITARY PSYCHIATRY

Work Unit 041 Behavioral Variables in Autonomic Function and
Disease in Military Personnel

Investigators.

Principal: Hursh, CPT S.R.

Associate: Faden, MAJ A.I., Cuthbert, CPT B.N.,
Hamilton, CPT B.E.

DESCRIPTION

Work within this unit directs the methodologies of both psychology and neurophysiology in multi-level studies of behavioral, systemic, and neural changes which precede or accompany the development of neurologic and psychosomatic disease. At one level, techniques of operant and respondent conditioning are employed as models of both phasic and chronic psychological and emotional stress while concomitant changes in the autonomic nervous system are monitored. These studies have focused on the control of autonomic responses by complex environmental conditions requiring either intense and extended behavioral involvement or exposure to noxious and stressful conditions. Measures of cardiovascular or gastrointestinal function provide clues to the development of disease.

At another level, physiologic and biochemical techniques are used to alter directly the autonomic nervous system while concomitant behavioral and physiological changes are monitored. These studies have focused on the neural pathways and neurochemical mechanisms which probably mediate the autonomic responses to these conditions.

ENVIRONMENTAL INFLUENCES ON THE AUTONOMIC NERVOUS SYSTEM

Environmental modulation of the cardiovascular system. Laboratory work within this area has been curtailed somewhat this year while limited personnel lend support to field studies of behavioral rhythms and effects of transmeridian desynchronization (jet lag). A summary of the contribution to that study conducted under this project is described below.

Most of the laboratory effort was devoted to initiating a computer-directed laboratory system to run simultaneously the experimental sessions and collect physiological and behavioral data in real time for 10 primate chambers. In addition to the behavioral programming, the software to date has the capability to record heart rate, blood pressure, respiration, and temperature on-line in any time frame desired. It is also possible to record

these measures continuously for 24 hours to study circadian patterning.

Currently monkeys are in different stages of training for four different experiments. Two of the studies are based on earlier findings, summarized in previous annual reports, that monkeys working for food on a behavioral schedule requiring low frequency, paced responding displayed unexpectedly large increases in heart rate and blood pressure during the task. The first experiment will compare such paced responding with less demanding tasks, using both positive and negative reinforcement, in an effort to assess the relative contributions of the task demand vs. the type of reinforcement in eliciting physiological response. The second study will compare paced responding for food with a vigilance task for sessions conducted at varying times of day. Changes in heart rate as well as behavior will be recorded in examining the interactions of the two tasks with differing times of day.

The other two experiments, being performed in conjunction with members of the Neuroendocrinology Branch, involve the endogenous opiate beta-endorphin. This recently discovered drug has attracted much attention both for its role in organismic response to stress and as a potential analgesic. In one experiment, injections of beta-endorphin or morphine are given to monkeys working for food on a behavioral schedule, and the effects on behavior and physiology noted. One pilot animal has been completed to date and the results summarized in last year's report. The other experiment will determine analgesic doses of beta-endorphin and morphine using a procedure called shock titration. In this paradigm continuous low levels of shock are regulated by the animal, so that changes in pain threshold or tolerance can be measured following drug injections.

All four of these experiments are currently in progress under the computer system. A separate study under way is designed to follow up aspects of the earlier experiments concerning the interactions of operant and respondent conditioning. Rats have been trained on a shock avoidance schedule, following which a stimulus sequence of a tone followed by food (a classical conditioning paradigm) will be presented during the avoidance sessions. The major independent variable will be whether or not a series of tone-food pairings was first presented outside of avoidance sessions. The aim is to evaluate the interaction of the classical conditioning and operant conditioning procedures by measuring changes in avoidance response rates during the tone. Broadly speaking, such experiments are directed towards

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an understanding of the ways in which ongoing performance may be disrupted by intrusions of various sorts.

Environmental modulation of gastric function. Work has started on a pilot study designed to evaluate the effects of chronic infusion of Pentagastrin (a synthetic gastric hormone) at graded doses on gastric secretion of acid. In this study two male rhesus monkeys will be adapted to living in loose restraint chairs and trained to accept naso-gastric tubes. After habituation to the experimental environment, each monkey will be prepared with a silastic catheter in a vena cava for automatic infusion of saline or Pentagastrin. The experimental procedure will involve alternating one week of infusion of Pentagastrin in saline with three week's infusion of saline alone. The week immediately prior to the infusion of Pentagastrin will be considered the baseline week for evaluation of the drug effect on gastric function. The measurement of the effects of the Pentagastrin will be done by sampling via the naso-gastric tube the stomach juice and adding via the tube a dye-marker, phenol red. By titrating the samples, the acidity of the juice can be calculated and by measuring the changes in the dye-marker, estimates of volume and emptying can be obtained. In this fashion, gastric dynamics (acid secretion rates, emptying volume, fluid secretion, etc.) can be monitored in response to the hormone and by measuring across time, the chronic effects. This information will allow a decision to be made as to the feasibility of using Pentagastrin to independently manipulate long-term acid secretion levels in another study designed to assess gastric dynamics in rhesus monkeys in response to stress.

Preliminary work has been completed in preparation for this second study designed to evaluate the stomach's response to the stress of noxious stimuli such as shock. The first aspect of it involves development of a technological means of chronically and automatically monitoring gastric function. The study involves the implantation of a pH electrode in the stomach along with attaching strain gauges to the stomach wall. The signals from these devices will be fed into an on-line computer for conversion from an analog signal to a digital one. In this manner, information about stomach function can be garnered over long periods of time, enabling newly developed statistical programs such as time series analysis to evaluate the data for periodic changes. This information will provide a baseline by which the effects of the noxious stimuli can be evaluated. In addition, by using female monkeys, the effects of the estrous cycle can be evaluated. It is hoped that this experiment will provide a useful tool for the chronic study of the stomach and will allow us to note the effect of stress, not only acutely, but for extended periods of time.

Field Studies of Response to Rapid Transmeridian Deployment ("Jet Lag"). (CPT Cuthbert assisted the Department of Military Medical Psychophysiology in collecting data summarized in the report of that department.) Most of two different months were spent in the field conducting two different studies (1,2,3,4). The remainder of the time was spent in preparing for the studies, directing the data analysis, drafting reports of the work, and preparing a new protocol appendix for simulation studies to be performed in an isolation chamber in the coming year.

During the first field study CPT Cuthbert served as director of a research team which collected more extensive data on a subset of the study population, and also directed the advance party of the WRAIR research team which deployed overseas prior to the subjects to enable data collection from the moment of the subjects' arrival. The second field study involved supervision of the research team collecting data from 120 soldiers deploying to Germany on four different aircraft as part of the REFORGER 79 exercise. This support of the Psychophysiology Department was necessary in order to meet strict time requirements imposed upon the research effort by CINCUSAREUR. The contributions from the Neurosciences staff included the following:

- 1) Experience in cognitive and performance testing of human subjects, to include selection and design of performance tests.
- 2) Computer facilities. To ease the heavy computing loads and meet deadlines for reporting the results, programs were written both for programmable calculators and computers and some processing was done on the PDP 11/03 in the Neurosciences Department.
- 3) Drafting experimental reports and manuscripts concerning the studies.
- 4) Experience in working with enlisted troops, and in carrying out research with human subjects.

AUTONOMIC VARIABLES IN DISEASE. (Studies of shock were carried out in conjunction with Dr. John Holaday of the Neuroendocrinology and Neurochemistry Branch.)

Endotoxin Shock. Preliminary studies, which were begun last year, were greatly expanded and extended (5,6). The initial demonstration that opiate antagonists reverse the hypotension caused by endotoxin administration has been confirmed in a large number of rats. Doses of naloxone as low as 0.1 mg/Kg have

produced a significant increase in mean arterial pressure and pulse pressure after endotoxin shock as compared to saline controls. Moreover, the cardiovascular improvement produced by naloxone in this model has been shown to be stereospecific, with only the (-) isomer of naloxone being efficacious. In addition, we have shown that intravenous (IV) β -Endorphin produces hypotension which is summative with that produced by endotoxin, and is reversed by (-) naloxone. Taken together, these findings suggest that endorphins are hypotensive factors in endotoxin shock and that the therapeutic effects of naloxone are specific and mediated by the opiate receptor (7).

In conjunction with Dave Reynolds' group at the University of Iowa, we have confirmed and extended the rat data in a canine endotoxin shock model (8). Following endotoxin administration, naloxone, in contrast to saline treatment, prevented the decrease in cardiac contractility, attenuated the decrease in mean arterial pressure and reversed the fall in cardiac output. Most importantly, naloxone treatment significantly improved survival.

Continuing studies are examining the mechanism of naloxone's effects after endotoxemia. These include glandular manipulation (hypophysectomy, adrenalectomy) and treatment with intracerebral-ventricular (IVT) naloxone administration.

Hypovolemic Shock. A successful hypovolemic shock model was developed, using conscious animals. Intravenous naloxone (1 mg/Kg) was demonstrated to significantly improve mean arterial pressure, pulse pressure and survival after hypovolemic shock (9). In conjunction with the University of Iowa, a parallel canine model was developed (10). Again, naloxone not only improved cardiac contractility, mean arterial pressure and cardiac output, but also dramatically improved survival. In fact, this model produced an LD 100 in saline treated dogs while the naloxone treated animals all survived.

Spinal Shock. Spinal shock was produced in anesthetized rats by transecting the spinal cord at C-7. Intravenous naloxone reversed the post-transection hypotension to baseline levels within 5 minutes, while saline administration was without effect (11). 48 μ g of (-) naloxone given IVT similarly reversed such hypotension, while neither (+) naloxone nor saline had any effect. These effects of (-) naloxone were shown to be mediated by the vagus nerves, and were completely blocked by bilateral vagotomy.

The ability of IV naloxone to reverse the hypotension caused by spinal transection has been confirmed in cats. Similarly, naloxone's cardiovascular effects were blocked by bilateral vagotomy (12).

Spinal Trauma. In cats, a successful model of cervical spinal trauma was developed, one that caused reproducible clinical and pathological changes. Preliminary studies have shown that significant hypotension is produced by the injury, and that these blood pressure changes are substantially reversed by opiate antagonists. Current studies are examining the possibility that treatment with opiate antagonists may limit the degree of injury following spinal trauma.

NEUROLOGIC STUDIES CONDUCTED IN COORDINATION WITH THE DEPARTMENT OF NEUROLOGY, WALTER REED ARMY MEDICAL CENTER

Pulmonary Neuropathy. A new polyneuropathy has been demonstrated in patients with moderately severe chronic obstructive pulmonary disease (COPD) (13). Individuals were studied prospectively using both clinical and electrophysiologic evaluation; all known causes of neuropathy were excluded in these patients. Of 23 patients studied, 18 had evidence of either clinical or subclinical neuropathy. Half of the abnormalities involved only sensory nerves, while half involved both motor and sensory nerves. No evidence of clinical or subclinical neuropathy has been found in the two control groups - (1) 10 normal young adults and (2) 10 age matched controls with minimal COPD. The findings from this study convincingly demonstrate the existence of a polyneuropathy associated with COPD. Given the incidence of COPD in the population, and the fact that over 50% of all polyneuropathies go undiagnosed, the description of this neuropathy is of substantial epidemiologic significance.

Autonomic Dysfunction Clinic. We have continued to see and evaluate patients with autonomic disease. Two new clinical entities have been discovered - (1) progressive isolated sudomotor dysfunction of central origin and (2) dominantly inherited urinary incontinence. Successful new treatments have been demonstrated for three rare entities - (1) erythromelalgia (Aldomet), (2) post-traumatic dysautonomic cephalgia (Dilantin) and (3) dominantly inherited urinary incontinence (Tofranil).

Project 3E/62771A804 MILITARY PSYCHIATRY

Work Unit 041 Behavioral Variables in Autonomic Function and
Disease in Military Personnel

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION*		2 DATE OF SUMMARY*		REPORT CONTROL SYMBOL DD DRA (AR) 1498	
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(U) Psychiatric Illness; (U) Military Adjustment;									
(U) Environmental Factors; (U) Social and Psychological Factors; (U) Stress									
23. (U) This unit examines the dynamics of those specific factors within military organizations and environments that conduce to psychiatric illness, operate to produce psychiatric casualties and lead to the generation of dysfunctional behaviors and decrements in military performance. These studies have direct relevance for the development of programs of intervention and prevention and the development of effective techniques for the minimization of psychiatric casualties.									
24. (U) The methods of clinical psychiatry, social and clinical psychology, social anthropology and field epidemiology are used to identify factors that generate psychiatric casualties, behavior dysfunction and performance dysfunction and decrement in order to modify such factors or the relationship between them.									
25. (U) 78 10-79 09 Data gathered in studies of Artillery Fire Direction Centers have been analyzed to determine factors that mitigate or contribute to stresses of continuous performance. Field assays have been carried out with troops of the 82nd Airborne Division and projected studies of health factors in relation to deployment and transition states have been developed and will commence in this fiscal year. Special emphasis is being accorded to the relationship of group structures to the stress response. Studies of fire fighters have been directed towards the development of models for chronic combat stress. Field studies of women in the Army and the socio-medical and medical psychological factors effecting both mental and physical health have been implemented and pilot studies are approaching completion. These studies will delineate factors of risk for female personnel. Studies of the relationship of group structure to physiology and the handling of stress in BCT/AIT and in deployed field units are under development and should commence in the coming fiscal year. A historical review of factors involved in the generation and prevention of combat psychiatric casualties have commenced. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sept 79.									

Project 3E/62771A804 Military Psychiatry

Work Unit 042 Military Preventive Psychiatry

Investigators.

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Description

Neuropsychiatric casualties have represented a major source of manpower loss in every armed conflict in which the United States Army has been involved. In times of peace the Army suffers significant personnel losses and costs as a function of behavioral dysfunctions, performance decrements, effectiveness deficits, psychosomatic illnesses, psychogenically based disorders and neuro-psychiatric diseases. Many of these losses and costs appear to involve predisposing risk factors that are parts of the general and human ecology of the Army. Unique aspects and demands of military life engender both strains and stresses that further the risk of the individual and the group for dysfunctional and ineffective behavior. The symptomatic and often costly responses to stressful events and factors in the military are in part determined by the health status and coping styles of the individual and in part by the social milieu in which stressful events are experienced. The interaction of the individual and group within this special set of ecological settings - ranging from the intense, life-threatening multiple stresses of combat to the daily stresses and strains of garrison and training - represent the central concern of this work unit. This unit examines the dynamics of those specific factors within the military organizations and environments that conduce to psychiatric illness, operate to produce psychiatric casualties, and lead to ineffectiveness, the generation of dysfunctional behaviors, and decrements in military performance.

Central to this concern is the study of how the Military social milieu organizes, shapes, reinforces and mitigates responses to stressful events. These studies have direct relevance for the development of programs of intervention and prevention and the development of effective techniques for the minimization of psychiatric and behavioral casualties.

1. Studies of Artillery Fire Direction Centers in Simulated Sustained Operations

Description

These studies were carried out jointly with the U.S. Army Research Institute of Environmental Medicine, Natick, Massachusetts and the Department of Military Medical Psychophysiology, WRAIR. The description of the studies was dealt with extensively in previous Annual Reports. Essentially four artillery fire direction centers from the 82nd Airborne Division carried out simulated sustained artillery operations under the observation of a number of investigators. Behavioral, physiological, and performance measures were taken. Patterns of verbal communication in a small Army work group were examined to determine if they are related to stress and performance in this sustained operation simulation. Stress was defined as time periods when demands placed upon the groups exceeded their capacity to respond effectively to these demands. Such excess demand (stress) was inferred during multiple fire missions (as evidenced by the groups simple inability to respond to many requests) and increasingly over time due to fatigue (as evidenced by progressively deteriorating performance and failure to complete routine tasks). Fatigue reduces the capacity of the group to respond effectively, and thereby elevates some previously routine tasks into "stressful" tasks. Communication was selected for analysis as its processing is vital to effective command and control.

Progress

Data analysis and interpretation has been completed. It was hypothesized that during multiple fire missions non-task related communication would drop out on the assumption that the group would have to consolidate its resources and reduce nonessential procedures during times of stress. It was anticipated that non-task related communications would decline over time as fatigue reduced group capacity and that total communication would decline over time. Only partial confirmation

was obtained of these hypotheses. There was, as expected, a significant reduction in non-task communication during the multiple fire mission. But total numbers of communications did not change over time, indicating there was no "conservation" as the groups became fatigued. A small but significant increase in non-task communication was found over time. This increase was not large enough to cause a significant increase in total communication. It might indicate an increase in the emission of group maintenance behavior, (e.g., joking, socially bonding transactions, etc.) perhaps at some decrease in effectiveness since increased non task communication begins at about 24 hours into the task.

A multiple complex demodulation analysis of each individual's communication data (for one group) was also conducted. This is a technique for partitioning a waveform (in this case derived from the pattern of task related communications), into its component parts, and relating patterns in those components to individual behavior. A considerable concordance between appearance of high frequency waveforms and the time periods when individuals were performing poorly was discovered. Should these results hold upon examination of other groups, this could provide a non subjective technique to evaluate stress that does not require individuals to stop their tasks and respond to questionnaires or interviews.

2. The Psycho-Social Aspects of Health and Illness of Women in the Army

Description

The purpose of this study is to describe the relationship between various psycho-social and organizational variables and the health status and effectiveness of performance and functioning of women in the Army. In 1973 a decision was made to increase the enlisted strength of women soldiers to approximately 50,400, many serving in non-traditional MOS's. The impact of such increases in the number of female personnel on health resources, the possible consequences for individual and unit mental health and integrity are, at present, difficult to ascertain. Recent data on psychiatric hospitalization rates shows that for 1971-73 the rate of episodes of psychiatric hospitalization for women was approximately twice that of males if drug and alcohol categories are excluded. Provisional analyses have shown that Army women had more days lost than men due to hospitalization and a greater non-effective rate in 1975

counting both physical and psychiatric hospitalizations. When gender specific illnesses were excluded, females continued to have a higher hospitalization rate than males. Similar results have been shown for the Navy. Past experience indicates that when mental health rates differentials exist for groups in the Army, major contributor factors are invariably to be found in organizations and units and in psycho-social factors - particularly the stress provoking ones found in the military environment. This study employs extensive work in the field to: determine if such factors exist; isolate and describe them; and develop preventive measures. The specific pilot research program designed to investigate in detail the health problems of female soldiers and how these compare with those of male soldiers and to produce a descriptive ethnographic study of women in the Army with specific focus on health is in progress. From the data collected in this preliminary investigation hypotheses are being developed for later definitive studies in investigating gender differences in the health of soldiers. The research effort is concentrated on enlisted members stationed at Ft. Meade, Md. The methodologies being used includes participant observation, in-depth questionnaires, health diaries and a study of outpatient records. Participants are selected from the following units: Kimbrough Army Hospital to include the Community Mental Health Activity (CMHA), the Headquarters Command, the 76th Engineer Battalion, the 85th Medical Battalion, the 519th Military Police Battalion. Participant observers are attached to the 519th Military Police Battalion and the Headquarters Command.

Progress

Data collection commenced in January 1979 and will continue through December 1979.

1. Health diaries have been collected and analyzed for 46 female and 44 male soldiers selected from traditional (administrative) and non-traditional (field) units. Findings were as follows:

a. No significant differences were found between male and female soldiers' conceptualization of illness.

b. Women soldiers reported significantly more health problems than did men. This was true whether or not genital related symptoms were included.

c. More health problems were reported by both sexes in non-traditional (for women) MOSs than in traditional MOSs.

d. While there were no significant gender differences in the overall number of symptoms reported women had more symptoms referable to the digestive and genital system and reported more psychiatric and neurological symptoms than did men. Men reported more symptoms referable to the musculoskeletal system and the respiratory system than did women.

e. There was a significant relationship between special events (did anything unusual happen today, good or bad?) and symptom occurrence. The result was equally strong for men and women.

2. Data has been collected, coded and is presently being analyzed in that part of the study dealing with clinical bias and the health of female soldiers.

3. In-depth questionnaires continue to be administered to study social networks and support networks as they relate to stress and health of the female soldier.

4. Participant observers have completed six months of observations, one in a "traditional" unit and the other in a "non-traditional" unit. Observations will continue through the duration of the project.

5. Preliminary data from the study indicated two additional areas worthy of exploration:

a. A need to deal specifically with the knowledge of attitudes about pregnancy, birth control, venereal disease, abortion and genital related medical problems among female and male soldiers. A questionnaire was developed, tested and distributed to men and women in both traditional and non-traditional units. The data is now being analyzed.

b. Another area of concern is that of achievement motivation in women. An instrument has been developed and will be distributed to women in both traditional and non-traditional units during the early part of October 1979.

3. Attrition Studies

Description

The U.S. Army is presently suffering a significant problem of first-term attrition. Depending upon the post, six to 12 per cent of individuals entering BT are discharged prior to the completion of their initial training. Another 30-40% of enlisted personnel are separated from the service prior to the completion of their first tour of duty. The attrition problem is a multi-faceted one involving psychological, medical, sociological, individual, organizational and stress response components. Present levels of attrition represent a chronic problem both in terms of financial costs, and maintenance of force levels and readiness of the Army. Initial studies are being formulated to look primarily at the relationship of stress and stress response to rates of attrition in basic station training, advanced individual training, and at the first duty station. During the past year a prolonged series of pre-study observations were carried out at a basic training post in the eastern U.S. (Ft. Dix). Based upon the data acquired, a series of studies are presently under development. This research will be carried out collaboratively by the Department of Military Psychiatry with the Department of Medical Neurosciences and the Department of Psychiatry, Uniformed Services University of the Health Sciences.

Progress

Pre-study observations and discussions were carried out over a four month period at Ft. Dix, New Jersey. Extensive informal discussions were carried out with the officers, cadre and drill sergeants of twelve training companies. One company was followed, via observation and informal discussion with both cadre and trainees, on a weekly basis throughout its training cycle. In three other companies both cadre and trainees were observed and informal talks were held at selected points during the training cycle. The materials gathered in the course of the pre-study and materials gathered by TRADOC in recent questionnaire studies of training and of stress and stress coping problems of drill sergeants, have been considered in the light of past studies of Basic Training and a series of hypotheses and strategies for research have been developed. These are currently being integrated into an initial research protocol. It is anticipated that all protocols will be completed and field studies commenced within the first quarter of the coming fiscal year.

4. Health Aspects of Deployment

Description

A number of sources have indicated that illness rates (as defined by clinic visits) vary as a function of military unit activity, especially deployment. These illness rates rise briefly but dramatically during the pre-deployment phase and often result in a number of individuals not deploying with their unit for training or testing. This has a serious potential impact on (1) readiness through decreasing training opportunities, and (2) medical facilities through increased patient load.

Illness and disease represent a major obstacle to deployment of the soldier. The medical literature clearly indicates that a broad range of psychological and social factors determine whether or not an individual will experience a symptom of illness and the action taken in response to that symptom. Deployment, even for brief periods, may represent a source of considerable stress to the soldier and his family. Epidemiological studies indicate that such stress can lead to increased rates of illness and disease. Furthermore, group norms in respect to illness behavior and the supports that an individual receives from his associates and family, play an important role in the determination of reactions to such stresses and thereby illness rates and deployability.

Research objectives:

1. To determine the effect of unit deployment on illness rates (as measured by outpatient sick call visits) of selected combat and combat support units.
2. To determine the role of psychosocial factors in deployment related illness of the soldier by:
 - a. Determining the individual's perception of deployment related stressors and how it relates to his illness.
 - b. Determining how group norms (derived from work and/or recreational group) affect individual illness rates during deployment.
 - c. Determining whether increased sick call at time of deployment is due to an increase in perceived illness or a change in the individual's evaluation of and response to symptoms.

3. To determine the role in the individual's illness behavior of problems experienced by the soldier's spouse or children.

4. To study the socialization process of the first-term soldier in his new unit and to determine how and when norms of illness behavior and response to categories of symptoms are learned.

Progress

The formal research protocol is being written. Informal contacts have been made with potential study units. This research will be initiated at the beginning of the next calendar year.

5. Conditions of Psychiatric Casualties Under Catastrophic Conditions

Description

A considerable amount of research effort has been dedicated to understanding the dynamics underlying the generation of psychiatric problems in combat. During periods when US military forces are not engaged in combat, such problems are not normally available for study. Disaster research has been considered as offering a possible model which seems to present an opportunity to study highly stressful situations and their psychiatric sequelae. If these stressful situations are analogous to military combat, useful generalizations to military populations concerning generation of psychiatric casualties might be possible.

Progress

Three general areas of consideration for such research are discussed. These are: 1. Empirical (which includes a determination of whether civilian disasters generate psychiatric casualties); 2. Theoretical (which includes discussion of the problems inherent in generalizing from civilian to military populations); and 3. Methodological (which includes problems inherent in disaster research).

1) Empirical Area

Almost all persons who directly experience a disaster or who closely identified with disaster victims suffer some form

of acute physiological or emotional stress response (Janis, 1951; Fritz and Marks, 1954), but the evidence suggests that those responses are of relatively short duration and do not usually result in chronic neurotic symptomatology. Most of the evidence suggests, in fact, that there may be an actual decrease in the incidence of all forms of emotional illness (Fritz, 1961).

Thus, civilian populations probably are not appropriate to use as models to study variables which relate to the generation of psychiatric dysfunction due to the expected small number of such casualties. Further, the timely identification of sufficient numbers of affected individuals for study might not be possible.

2) Theoretical Considerations

A. Primary Motivation: A civilian is likely to be concerned with search and rescue, with rebuilding beginning very quickly. A soldier is likely to only have to continue his job ("soldiering" or fighting). Motivation to get started and keep functioning, with personal property and family comfort involved, might be quite different for the civilian and the soldier. The effect of "doing something for myself" could have a profound influence on the state of individual psychiatric functioning.

B. Trained responses: Civilians generally have no pre-specified job or task, although most soldiers would. Comparing responses of a population whose lives have been totally disrupted and who must learn entirely new (albeit temporary) behavior patterns, with those of a population which has a "trained response" might not be methodologically sound. It is unknown whether these "trained responses" and the presence of a relatively well-defined job and organization help protect the soldier against certain types of breakdown.

C. Medical channels and secondary gain: It is likely that some of the chief determinants of severity and persistence of psychiatric symptoms are the potential consequences of such symptoms. For a soldier continuation of symptoms may lead not only to permanent removal from combat but a medical pension as well.

The civilian may be better off staying in the impact area, where he can salvage personal goods and property, and aid and commiserate with neighbors and friends. Thus the secondary gain of symptom formation and special treatment (including

escape) probably does not play an important role in the genesis of behavioral problems in a civilian population.

D. Social support system: The civilian is likely to be in the immediate area of family and work, but strongest ties for the soldier might be "back home". The nature of combat soldiers' social support systems is likely to be qualitatively and quantitatively different from that of civilians.

E. Demographic variables: Age and sex are important correlates of psychiatric dysfunction. They affect both the probability that a symptom will be expressed and the prognosis of treatment. Soldiers are young and male, and quite atypical of the population in general.

3) Methodological Problems

There is a considerable array of methodological problems which must be dealt with to effectively conduct disaster research.

A. Our primary data would be retrospective.

B. Sampling techniques are likely to be haphazard and unrepresentative to general confusion and presence of outsiders (Fritz and Mathewson, 1957).

C. Emotional involvement with victims is likely to cloud the investigator's objectivity and to introduce additional bias. This can be overcome but requires extensive training and some considerable amount of interviewer experience. Refresher training would likely be continually required.

D. Disaster areas are frequently hard to reach physically, and transportation may be disrupted both by damage and convergence of crowds to the disaster area (Fritz and Mathewson, 1957).

Summary: The disaster literature indicates that the incidence of psychiatric dysfunction (beyond transient and mild acute effects) in disaster struck populations may be so low as to make systematic large sample studies unfruitful. Even if a sufficient number of such casualties could be identified and contacted there exist a number of theoretical problems to be resolved before results could be meaningfully generalized to military populations. These considerations along with the formidable practical difficulties of such work, suggest that

study of civilian disasters would be neither feasible nor cost effective.

Project 3E/62771A804 Military Psychiatry

Work Unit 042 Military Preventive Psychiatry

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION#		2. DATE OF SUMMARY		3. REPORT NO. (OF SYMBOL)	
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30. TECHNICAL OBJECTIVE, 31. APPROACH, 32. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code)									
23. (U) To examine neuroendocrine and neurochemical correlates of stressors specific to the military environment. Types of stress to be studied will include shock extremes of heat and cold, psychological stress, as well as desynchronization of circadian rhythm and continuous performance.									
24. (U) Laboratory and field studies will examine the neuroendocrine response to psychological stressors, ambient temperature extremes, transmeridian desynchronization, and continuous performance. These responses will be correlated with simultaneously-obtained data on performance decrement in the same subjects and work/rest schedules. Hormonal responses will provide bases for inferences concerning central nervous system neurotransmitter pathways essential to adaptation to stress, and optimization of work/rest schedules. This information is used to recommend pharmacologic and other therapies. Includes studies of physiological effects of hormones as well as assay development.									
25. (U) Further studies have shown that naloxone reverses hypotension in rats subjected to hypovolemic or spinal shock and significantly improves survival in hypovolemic shock. In dogs subjected to endotoxic or hypovolemic shock, naloxone improved left ventricular contractility, cardiac output, and stroke volume and 24-hour survival was significantly improved in both groups. Studies are continuing on the effect of contingency management on hormonal indices of stress and group behavior under continuous performance requirement. Preliminary results in 2 groups suggest that 24-hour urinary testosterone correlates positively with dominance/submissiveness behavior in a subject added in mid-experiment to an already established social situation. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.									

Project 3E/62771A804 MILITARY PSYCHIATRY

Work Unit 044 Neuroendocrine Response to Military Stress

Investigators.

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Associate: G. Jean Kant, Ph.D., J.W. Holaday, Ph.D., MAJ
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Our approach emphasizes patterns of neuroendocrine response rather than the potentially misleading study of single neuroendocrine systems. A major immediate goal is to define in detail the characteristic hormone response profiles for various stressful stimuli: effect of multiple stressors, environmental factors such as continuous performance requirements, transmeridian desynchronization and/or ambient temperature, biological factors such as differences in sex or age, or effects of pain or neural injury. Understanding the impact of these factors often requires laboratory experiments which pursue leads developed in field studies and support work in the Departments of Military Psychiatry and Military Medical Psychophysiology. Hormonal response to stressors, for example, which can be observed in both field and laboratory studies, provides a critical link in facilitating coordination between those two efforts. Studies of neurochemical, neurophysiological and neuroanatomical regulation of the body's response to stress provides a means of interpreting the field data. Rational design of prevention and treatment regimens for neuropsychiatric illness induced by military stress requires interpretation of the hormonal response profiles for stress in terms of the neurochemical systems producing the response. Hormone profiles are measured in clinical and animal studies as indicators of stress; they provide a basis for inferences about changes in brain function. For example, plasma prolactin levels provide a sensitive indication of the degree of central dopaminergic blockade induced by antipsychotic medication (1). Growth hormone secretion is stimulated by central catecholaminergic systems and is markedly increased during slow wave sleep (2,3,4). Thus, biochemical measurements may provide a useful measure of adequacy of rest periods during continuous performance studies. Additional neurochemical studies in animals will directly examine effects of stress in neurochemical activity of the brain and explore physiological, dietary, and pharmacologic methods for preventing stress-induced psychologic and physiologic disease.

A new and increasingly important area of research concerns the role of pituitary and brain peptide hormones in adaptation to

environmental stressors. Most promising are peptide subunits of beta lipotropic hormone (β LPH). β LPH was isolated from pituitary glands and characterized by Li in 1964 (5). Its major function was thought to be lipolysis and liberation of free non-esterified fatty acids. Recently, however, interest has focused on a LPH peptide fragment composed of amino acids 31 through 91 of β LPH. This peptide has been named Beta-endorphin (β E). It is a very potent analgetic and hypothermic agent. Immunocytochemical studies have demonstrated intense staining for both α endorphin (α E) and β endorphin (β E) in the pars intermedia of the rat pituitary, with discrete cells staining positively in the anterior pituitary as well and with no staining in the pars nervosa (6). While the β endorphin antibody was highly specific for that peptide, the β endorphin antibody cross-reacted considerably with β lipotropic hormone (β LPH), which was previously demonstrated to be present in the pars intermedia and pars distalis (7). The function of the pars intermedia is not known with certainty. It has been associated with melanocyte stimulating hormone (MSH) (8), adrenocortical stimulating hormone (ACTH) (7) as well as β LPH (7). We are interested in assessing the role of the pituitary as the source of circulating endorphins and investigating the interrelationship among endorphins and hypothalamic, pituitary, and adrenal hormones. It is known that endorphins are found in the pituitary gland and in brain, they possess antinociceptive activity and we believe they may be involved in thermoregulation and cardiovascular shock as well. Several important questions remain to be addressed. Can endorphins be measured in blood? Are they released from the pituitary? If so, what regulates their release? What are the physiological and psychological roles of the endorphins, if any? The potential relevance of these questions to military medicine is illustrated by the data of Beecher (10) who studied the management of pain in soldiers wounded in combat in WWII. Of 215 severely wounded soldiers, 32.1% reported no pain and 25.6% only slight pain. Are endorphins released in response to trauma or pain?

It has long been recognized that the cardiovascular system is exquisitely sensitive to the effects of exogenous opiates (11,12). In rats, less than one percent of the morphine dose necessary to produce antinociception results in significant hypotension and bradycardia (13). The endogenous opiate, Met-enkephalin, also has potent hypotensive effects (14,15). It is now known that another endogenous opiate, β Endorphin, is concomitantly stored and simultaneously regulated along with pituitary adrenocorticotropin (ACTH) (16,17) since stressors appear to result in the release of both peptides (16,17,18), it appears possible that stress-induced release of β Endorphin may result in alterations in blood pressure. We have now demonstrated that intravenous β endorphin produces hypotension.

The administration of bacterial lipopolysaccharide endotoxin in animals has often been used as a model of human septic shock (19,20). It has been suggested that the profound hypotension induced by endotoxin is mediated through the release of endogenous substances (19,20). We hypothesized that if endorphins were functionally released by endotoxins and involved in the hypotensive effects produced by endotoxins, then blockade of endorphins should reverse such hypotension (21). To test this hypothesis, we employed naloxone, a specific opiate antagonist, in studies of blood pressure changes following endotoxin administration to conscious rats. Our results demonstrated that naloxone not only rapidly reversed endotoxin-induced hypotension, but also prophylactically blocked its occurrence.

Of greater immediate importance to battlefield casualty management and care, we studied the effects of naloxone in a conscious rat model of hemorrhagic shock as well as following cervical spinal cord transection (spinal shock) in unconscious rats (22,23).

In the rat model of hemorrhagic shock, rats were bled through a chronically indwelling intravenous cannula until they reached a mean arterial pressure (MAP) of approximately 40 mm Hg. After being maintained at this hypotensive level for 20 min. by appropriate adjustments in the amount of blood removed, rats were treated with equivalent volumes of either saline ($n = 15$) or naloxone (1 mg/Kg, $n = 15$), and blood pressure was monitored for an additional 2 hrs. The naloxone-injected group showed a rapid improvement in MAP and pulse pressure which persisted at significantly higher levels than observed for saline-treated rats over the first 1-2 hours following injections. Moreover, 24 hour survival was significantly improved in the naloxone-treated group.

Since spinal cord transection is known to produce a condition termed "spinal shock" which is characterized in part by hypotension, we investigated the effects of naloxone in this pathological state as well. Following laminectomy at the cervical region, a suture was placed extradurally around the cord at the level of C6-C7. As before, rats were affixed with an intravenous cannula and tail-artery cannula. In addition, a number of rats were provided with ventricular transcranial guide tubes which allowed for intrathecal administration of drugs directly into the right-lateral ventricle. Following cervical cord transection by a sharp pull on the ligature in these anesthetized rats, MAP and PP dropped precipitously. Subsequently, (-) naloxone (10 mg/Kg) or an equivalent volume of saline were administered intravenously. In the rats with intracranially implanted ventricular guide tubes, either (-) naloxone (48 μ g), (+) naloxone (48 μ g), or water vehicle were injected. An equivalent improvement in MAP and PP was seen following

(-) naloxone administration intravenously or intraventricularly. By contrast, the (+) stereoisomer of naloxone (which is pharmacologically inert) as well as drug vehicles were without effect on these measures.

These findings not only provide evidence of an endorphin involvement in the pathophysiology of yet another shock model (spinal shock), but also provide important insights into the mechanism of action of naloxone in producing its therapeutic benefits. The (+) stereoisomer of naloxone, which is known to be pharmacologically inactive at opiate receptors, is also inactive in improving MAP and PP in spinal shock. This indicates an involvement of endorphins at opiate receptor sites. Moreover, since injections of small amounts of (-) naloxone directly into the ventricles provide quantitatively and qualitatively similar results in improving MAP and PP, this indicates that the endorphinergic involvement in the pathophysiology of spinal shock is probably mediated through cardiovascular regulatory centers in the central nervous system. Finally, since vagotomy blocks the effects of (-) naloxone in spinally transected rats, evidence is provided to support our hypothesis that endorphinergic systems mediate their cardiovascular effects through parasympathetic, vagal inputs into the heart.

In collaboration with Dr. David G. Reynolds, Director of Surgical Research, University of Iowa Hospitals and Clinics, we have completed additional studies on canine endotoxic and hemorrhagic shock. These experiments confirmed and extended rat studies since it was shown that naloxone affects its increase in blood pressure through increasing cardiac contractility, not through an alteration of peripheral resistance.

In pentobarbital-anesthetized dogs, Swan-Ganz catheters were implanted through the femoral vein to allow for measurements of pulmonary wedge pressures as well as cardiac output by the thermodilution technique. Dogs were then given either endotoxin or subjected to hemorrhage at 40 mm Hg for 1 hr. As in the rat studies, (-) naloxone (2 mg/Kg) or an equivalent volume of saline was then administered intravenously. Left ventricular contractility (measured by means of a pigtail catheter inserted into the left ventricle), cardiac output, and calculated stroke volume were significantly improved only in the naloxone-treated dogs. By contrast, venous return, pulmonary wedge pressures, and calculated total peripheral resistance were unaffected by naloxone. These data were obtained in both dog models of shock and indicate that naloxone produces its therapeutic benefit through improving cardiac performance as opposed to an involvement of improved venous return or altered peripheral resistance. Moreover, in both of these dog

models, survival at 24 hours was significantly improved by naloxone.

Thus, these studies on rats and dogs not only demonstrate a possible pathophysiological role for stress-induced release of endorphins, but also point to the importance of narcotic antagonists such as naloxone in battlefield care and management of shock states. In summary, investigations on the site and mechanism of endorphin involvement in shock pathophysiology reveal that these effects are probably caused by endorphins which subsequently affect parasympathetic centers in the central nervous system to increase vagal tone and thus decrease cardiac performance. Current research, which is presently underway, suggests that the endorphins which produce these deleterious effects are of probable pituitary origin.

In collaboration with CPT Bruce Cuthbert of the Physiology and Behavior Branch we have completed a preliminary study of the effects of intravenous injection of beta-endorphin on respiration, heart rate, blood pressure and operant performance in the Rhesus Monkey. Marked hypotension and tachycardia were observed and fixed-interval and fixed-ratio operant performance were briefly disrupted. A dose of phentoamine which produced similar hypotension and tachycardia failed to disrupt operant performance. Respiration rate and amplitude were unaffected by beta-endorphin in doses of 5 and 20 mg. Beta endorphin has been shown to have analgetic potency (24). We have an approved protocol to compare the effect of analgetic doses of beta endorphin and morphine on respiration, blood pressure and heart rate in monkeys, using a shock-titration technique. If βE produces less respiratory and circulatory depression than equianalgetic doses of morphine, βE might be preferable in some instances as a clinical analgetic.

Collaborative studies with Dr. Joseph V. Brady at Johns Hopkins University have been examining the effect of contingency management on performance on a group task continuously performed over many days. This experiment studied the effects of two incentive conditions, i.e., positive and negative reinforcement, on individual and social behavior within a three-person group residing in a programmed environment. Such effects were evaluated by comparisons between conditions of work-task productivity, ratings of interpersonal adjustment and of intrapersonal "mood" status, e.g., depression, and other measures. Performance was monitored on the Alluisi Multiple Task Performance Battery within a duty station which could be occupied by subjects one at a time on a self-determined rotational basis continually over a 24 hour period. Heart rate, skin potential, frontalis EMG, and skin temperature were recorded during work, and total 24 hour urine volume was collected for analysis of testosterone

and cortisol levels. The substitution of aversive for appetitive control produced lowered productivity, episodes of disruptive behavior, reports of depression and anger at experimenters but no changes in urinary free cortisol in preliminary experiments. Mean daily urinary free cortisol levels did correlate positively however, with mean daily scores on the multiple task performance battery (five concurrent tasks). Further studies have been carried out examining the effect on performance and hormone excretion of the addition of a group member in mid-experiment. Preliminary results in two groups suggest that 24 hr urinary testosterone correlates positively with dominance/submissiveness behavior in a subject added in mid-experiment to an already established social situation. An analysis of testosterone levels obtained from the 24 hr total urine volume collected during experiments showed inverse relationships to the experimental manipulations of size and composition of a group. In the first group the testosterone levels of established 2-person group members dropped when the novice member was introduced and then returned to baseline levels when he was withdrawn. Significantly the novice member's testosterone levels were consistently elevated in comparison to his team mates and he also showed corresponding elevations on the "Dominance" factors associated with the pre-mission screen battery. In the 2nd group, the novice team member showed diminished testosterone levels and he also showed the lowest value on the pre-mission "Dominance" scores. These observations show the influence of personal history (ie, individual difference variables) and role differential as assessed from behavior, physiology, and psychometric perspectives on the potential personal re-adjustments and inter-personal challenge that a group must manage successfully when a change in membership occurs. The introduction of a third subject into the experimental situation did not affect secretion of urinary cortisol.

A study of basic training at Fort Dix has been initiated in collaboration with the Departments of Psychiatry, Division of Neuropsychiatry and the Uniformed Services University of the Health Sciences. As a result of preliminary surveys, the Drill Sergeants have been selected as a population for further study, having shown symptoms indicative of a high degree of job-related stress. A study is being planned incorporating biochemical measures to quantify the endocrine response to stress in this population.

In collaboration with the Nephrology Service, WRAMC, and the Department of Military Medical Psychophysiology, a member of our staff is using solid state actigraphs to study 24 hr activity patterns in renal failure patients on hemodialysis or following successful renal transplantation. The purpose of these studies is to provide objective evidence of the degree of rehabilitation

provided by transplantation as measured by activity levels. It should be feasible to use this technology in assessing rehabilitation in other disease states.

Additional studies have been planned with the Department of Military Medical Psychophysiology on the hormonal indices of trans-meridian desynchronization and effects of prophylactic measures.

Project 3E/62771A804 MILITARY PSYCHIATRY

Work Unit 044 Neuroendocrine Response to Military Stress

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Patents:

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<p>23. (U) To identify factors in the military organizational, social, psychological and physical environment that create risk for and conduce to psychiatric breakdown, behavioral dysfunction, psychosomatic and physical illness as they impact on individual and unit effectiveness and consume health care resources.</p> <p>24. (U) The methods of epidemiology, including records analysis, population and demographic analysis, questionnaire and field and cohort studies as well as observational methods are employed to develop requisite data.</p> <p>25. (U) 78 10 - 79 09 During this period efforts centered on three areas identified by commanders as important concerns within the European theater. These were (a) biomedical aspects of continuous performance, (b) epidemiology of administrative discharges and (c) "psychological autopsies" of drug overdose victims. It was found that morale was generally low despite concentrated efforts to remedy sources of soldier unhappiness. In studies of the epidemiology of administrative discharges, the detailed demographic profile of those studied was considered remarkable primarily for its real lack of distinguishing features. The absence of a high school diploma was the best distinguishing feature of the demographic variables but even in that instance less than half of the sample had dropped out of school and 25 percent of the control group had also done so. These studies suggest high levels of attrition are inevitable without major changes in all phases of Army life, increasing emphasis on providing soldiers' social as well as physical support. Preliminary studies of drug overdose victims indicate neither personal nor unit characteristics distinguished the victims. No pattern of organizational climates was evident; both task - and people-oriented commanders with either prominent or low key drug suppression programs experienced overdose incidents in their units. For technical report see Walter Reed Army Institute of Research Annual Report 1 Oct 78-30 Sep 79.</p>							

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Project 3E/62771A804 MILITARY PSYCHIATRY

Work Unit 046 Medical Factors Limiting Soldier Effectiveness

Investigators.

Principal: Ingraham, MAJ L.H.

Associate: Manning, MAJ F.J.

Description.

This field unit, stationed in West Germany with the US Army Europe and Seventh Army, identifies and investigates physical, psychological, social, and organizational factors bearing on individual and unit performance and combat readiness. Initial efforts have focused on three areas identified by commanders as important concerns within the European theater.

BIOMEDICAL ASPECTS OF CONTINUOUS PERFORMANCE

A central Soviet Army doctrine calls for continuous combat "night and day, without let-up, until the enemy is defeated." Until very recently, the implications of this for the US Army were felt to be primarily technological, centering on the need for more and better night vision devices. In summer of 1977, however, Fifth Corps Artillery approached WRAIR with a request for information on the ability of their soldiers to endure the physical and psychological stress they would face in the sustained high intensity combat their planning scenarios outlined for Europe. They hoped, in addition, for some indication of leadership traits essential in maximizing performance under such conditions. Experimental manipulations were of course not possible, but extensive observations of a typical cannon battalion were made over the course of a seven month period, both in garrison and during field training. Both active duty soldiers and their families were included in these observations, which were supplemented by interviews, questionnaires, personnel and medical records screens, and examination of third party evaluations like the annual Army General Inspection and Nuclear Surety Evaluation. The general strategy was to examine the applicability of well-controlled laboratory findings to this specific real-world situation, and to discover possible "fault lines" along which such a unit might crack under the stress of continuous operations.

Findings can be organized under four general headings. In the area of Fatigue and Performance, it was clear that in the field, as in the laboratory, it was psychological rather than physiological exhaustion that is the critical problem with sleep deprivation. Decision-makers, and others whose jobs are primarily cognitive, not only are more affected by lack of sleep, but precisely because they are in responsible positions, get significantly less sleep than those with more labor-intensive jobs. They also have most difficulty recognizing and/or admitting the decrements in their own performance, which differ in kind from those of the heavy laborers. The latter do less work as they grow fatigued, the former do worse work, though in both cases it is self-paced or self-initiated work rather than transient responses to external demands which is affected.

Many of the factors historically associated with high rates of neuropsychiatric casualties are present in current combat scenarios: initial contact by green troops, intense artillery and aerial bombardment, high casualty rates, defensive operations, poor communications, physical fatigue. We found, in addition, widespread scepticism about plans for evacuation of families (in an increasingly married Army), little chance for soldiers to develop close interpersonal bonds due to high turnover rates, and a very clear-cut vertical segregation of enlisted, NCO, and officer ranks, characterized at each level by lack of respect for and confidence in members of the other two levels. Historical data suggest that these factors too are associated with high levels of neuropsychiatric casualties.

A friendly and relaxed leadership style, exhorting and reminding rather than ordering, seemed to work best with tired soldiers, though tired leaders unfortunately tend to opt for the less demanding autocratic style as fatigue increased. Emphasis on "zero defects," the belief that one mistake ends a career, an adversary relationship between tester and tested, and personnel and training policies apparently overrating short-term achievement were all identified as problem areas for leadership. Consequences were that officers assumed the jobs of (possibly) less competent subordinates, competent soldiers were assigned more and more of the work of their less competent peers, training centered solely on the next evaluation, cross-training was ignored, and command universally viewed as an impossible job, to be endured only as long as advancement required.

Morale was generally low, despite concentrated efforts to remedy sources of soldier unhappiness throughout Germany during the course

of the study. It is suggested that this is because remedial efforts on personal problems, while the major unmet need, among young single troops especially, is social. Acceptance in a mutually supportive group, crucial in combat, and apparently in peace, was constantly sought. Rotations, details, centralization, and the verticle segregation cited above made it difficult to form such groups along organizational lines. Race and drugs then served as axes around which such groups would form instead. Those unwilling or unable to join such groups (i.e., NCO's and officers, married soldiers and their wives) then found themselves without the social support which might otherwise make living in a strange country far from home and family easier to bear.

The study concludes with a list of concrete recommendations for change.

EPIDEMIOLOGY OF ADMINISTRATIVE DISCHARGES

A continuing and very serious problem to the US Army, not only in Europe, but world wide, is the very high rate at which apparently fully qualified enlistees fail to complete their first enlistment. Attrition, as it is often called, is over 40% when washouts from basic training are included. In Europe, a battalion a month goes home on "administrative discharge." Nearly all of these stem from some form of maladaptive behavior, e.g., continuing drug or alcohol abuse, lack of motivation, attitude, or self-discipline, inept duty performance, or simple inability to adapt socially or emotionally (An additional company a month is incarcerated or otherwise lost to the command for criminal behavior). In an effort to find out exactly who ends up in this lost battalion, and why, investigators screened the personnel records of all soldiers leaving Europe for administrative reasons during one entire month (N = 649). In addition, in-depth interviews were conducted with a 10% sample, focusing on military careers, from enlistment motivation to feelings about leaving the service.

The detailed demographic profile of these discharges of April 1978 is remarkable primarily for its real lack of distinguishing features. Compared to the total junior enlisted population in Europe at that time (pay grades E1-E4), they contain about the right proportions of blacks and women, married and single. They come in a variety of job fields, including combat arms in about the quantity that might be expected on a purely numerical basis. Food Service sticks out as the only career field contributing

disproportionately, and only 13 came from jobs which required skills other than their primary specialty. Indeed they were slightly younger than the average private, and had spent slightly less time in Europe and in the service, but these differences were slight, and in a way predetermined by our selection criterion of premature discharge. As in a number of earlier studies, absence of a high school diploma was the best distinguishing feature of the demographic variables, but even in this instance, less than half the sample had dropped out of school, and 25% of the control group had also done so.

Those we interviewed were no more unusual. Almost all had enlisted assuming they would return to the civilian job market with coveted skills. Some had no concept at all of what to expect in the Army, and now they were leaving, angry with the Army, but happy to go. A much larger group was disappointed, both with the Army and with being discharged. They had enlisted with far fewer illusions, expecting discipline and hard work, but events just had not worked out that way. Basic training was cited almost across the board as the highlight of their service. They liked the structure (knowing what was expected of them every minute), the obvious good planning and the feelings of accomplishment and camaraderie they had felt. Socially, our subjects presented themselves as alone and scared, though none used those terms. Few reported any positive feelings toward their units at all, and the constantly recurring theme in talking to these soldiers, or ex-soldiers, was that of an uncaring leadership, insensitive to human problems, concerned only with mission completion.

These data have important implications for personnel policy, suggesting that while expanding the role of women in the Army may help maintain strength levels, it will do so only by enlarging the recruiting pool, not by cutting attrition. Similarly, more and better bonuses for recruits may not be helpful, if in fact "gaining job skills" is the primary enlistment motivator. Finally, the lack of distinguishing features in our sample might be taken to mean high levels of attrition are inevitable without major changes in all phases of Army life, increasing emphasis on providing soldiers social as well as physical support.

"PSYCHOLOGICAL AUTOPSIES" OF DRUG OVERDOSE VICTIMS

The purpose of this exploratory research is (a) to describe the victims of drug overdose; (b) to describe the unit in which overdose casualties appear, and (c) to discover means of preventing such casualties. The method used is the psychological autopsy technique

wherein investigators visit the site of the drug overdose, interview leaders, friends, and associates of the victim, and attempt to piece together the circumstances of the overdose. Data collection began in July 1978 and will continue until July 1980.

Because the technique had no precedent in the literature, attention focused on victims admitted to the hospital and placed on the Seriously Ill lists. Some of these victims recovered; they provided a check on the kinds of information unavailable from those who died. Of the 16 cases investigated as of 1 July 1979, 12 recovered and 4 died.

Preliminary results indicate neither personal nor unit characteristics distinguish the victims. No pattern of organizational climates was evident; both task- and people-oriented commanders with either prominent or low key drug suppression programs experienced overdose incidents in their units. The "typical" drug overdose victim was invariably male, E-3, 4, or 5, about 21 years old, from any ethnic group, and equally likely among combat, combat support and combat service support units.

The victims were good soldiers, not the marginal and ineffective. Their leaders regarded them as average to superior in their military job performance; four of the 16 were being considered for promotion at the time of their accident; only two had adverse actions pending against them at that time. One continued serving as a valued section chief while awaiting his Chapter 9 discharge.

Nor did the victims tend to be barracks rats. Most got out of the barracks, many had local national girlfriends and several were active in company athletics. They were not new arrivals in Europe; time in country ranged from 4 to 37 months with an average of 24 months in the country.

Only two of the 16 were addicted to heroin. The others used heroin intermittently at parties and other special occasions for approximately 6 months prior to their overdose.

With respect to the fatal or near-fatal doses, there is no evidence they were either more potent or of greater quantity than the victim was accustomed to using. The typical overdose sequence included heavy drinking with buddies before returning to the barracks for a nightcap of heroin, either injected or sniffed through the nose. In the next few hours they passed out, vomited, choked and sometimes died. Prevention is complicated by the use of such unhelpful home remedies as cold showers, or by delaying

calls for help until evidence of drug use and witnesses to the accident can be removed from the scene.

Despite the fact that five cases were enrolled in the Community Drug and Alcohol Assistance Center, who will overdose when appears unpredictable. Of the 16 cases, 12 were totally unexpected by practically everyone in the company including the wife of one victim, three were unexpected by the leadership but not surprising to their work groups, and one surprised nobody.

Data collection continues until July 1980. Additional personnel enable inclusions of Dead On Arrival cases as well as the seriously ill and very seriously ill patients. In addition, we are reviewing the records of all drug overdose death cases during the calendar years 1978 and 1979. The final report will include field investigations as well as appropriate comparisons with both the death records and the USAREUR Personal Opinion Survey profiles of drug users.

Project 3E/62771A804 MILITARY PSYCHIATRY

Work Unit 046 Medical factors limiting soldier effectiveness

Literature Cited.

Publications:

1. Ingraham, L.H. The Myth of Illicit Drug Use and Military Incompetence. Medical Bulletin, 36: 18-21, 1979.
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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1 AGENCY ACCESSION*	2 DATE OF SUMMARY*	REPORT / CONTROL SYMBOL DD FORM 1498 (AR) 636	
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23. (U) This unit examines military organizational, social, psychological, and environmental factors that create risk for and conduce to psychiatric disease, psychosomatic illness, behavioral dysfunction and physical illness as they effect Army personnel and impact on care giving agencies.								
24. (U) The methods of epidemiology, including records surveillance, population and demographic analysis, questionnaire and field and cohort studies as well as methods of the psychological and social sciences are used to delineate environments of risk for psychiatric illness and periods of special risk for such illness at critical points in the career of the soldier.								
25. (U) 78 10-79 09 Analysis of the past and present patterns of psychiatric disease, illnesses with a psychosomatic component and behavioral dysfunctions is underway utilizing materials available through IPDS and other DA reporting systems. Studies are being carried out analyzing the psychological and health problems of women in the Army. Studies are being carried out on the epidemiology of neurological syndromes following penetrating wounds. Further studies of factors involved in suicide of military personnel are underway. Analysis of cohorts representing selected accessions of personnel during FY72 to determine precursors of dysfunctional behavior continues. Data gathering for the development of an instrument to study the relationship between social supports and health has been completed, analysis and development will continue during the coming year. Pre-study surveys have been carried out on the relationship of stress to attrition to BCT/AIT. Formal studies will commence during the coming fiscal year. Studies of socio-medical and medical-psychological consequences of deployment are in the process of development. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sept 79.								

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Project 3E62771A804 Military Psychiatry

Work Unit 047 Military Psychiatric Epidemiology

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Description

The military environment places demands and strains upon its population that are markedly different from those of civilian environments. The demands and differences in terms of individual and unit effectiveness and performance, mental and physical health, and behavioral disruption and dysfunction have chronic effects in peacetime. In periods of deployment and combat, such stresses may have acute affects on the capability of units and individuals to perform their missions. This unit examines military organizational, social psychological, and environmental factors that create risk for and militate against psychiatric disease, psychosomatic and physical illness, behavioral dysfunction and disruption of performance as they affect Army personnel and impact on care giving agencies. The methods of epidemiology, including records surveillance, population and demographic cohort studies and methods of the psychological and social sciences are used to delineate factors conducing to risk as well as mitigation for such illnesses, disruptions and dysfunctions.

1. Battle Injuries and Associated Neurological Disorders Sustained by Active Duty Army Personnel During the Vietnam Conflict.

Description

A descriptive analysis of battle injuries sustained in previous conflicts may be of value in predicting the effects of

combat on health and recovery patterns and its long term impact on the Army Medical system. A research proposal has been implemented to perform an epidemiologic study of battle injuries and associated neurologic disorders sustained by active duty Army personnel during the Vietnam conflict.

Progress

Causalgia is the painful and hypersensitive state which occurs subsequent to peripheral nerve injury. Military medicine, with its concentration of battle-wound traumas, has been the primary source of cases and information about causalgia. Mayfield (5) has reviewed the literature through WWII and has summarized the history of wartime causalgia. He concludes from the case-series results reviewed that causalgia occurs in 2%-4% of all peripheral nerve injury cases when seen in general hospitals several weeks after wounding. Based on the observation that spontaneous recovery does occur and that one series (from a Burma evacuation hospital) reported a 16% rate for cases seen shortly after wounding, Mayfield suggests that the occurrence of causalgia as a consequence of peripheral nerve injury may be underestimated due to the delay in delivery of definitive medical care. The analysis of the epidemiology of causalgia among soldiers wounded in Vietnam allows the opportunity to examine the incidence question further and generates speculation concerning diagnostic and physiologic mechanisms which may be determining considerations in the occurrence of causalgia in peripheral nerve injuries.

The data are derived from records of the Patient Administration System of the Army Surgeon General. The Individual Medical Record (IMR) from 1964 through 1970 and the Individual Patient Data System (IPDS) from 1971 through 1973 were used to obtain extracts of the inpatient hospitalization discharge records summary in standard format. The records of interest were defined by the International Classification of Diseases, Revision Eight (ICDA-8), Codes of 3559 for causalgia ("other and unspecified neuritis or neuralgia") and 952-957, 959 for peripheral nerve injury. Since 3559 is the code used within the ICDA-8 for other diagnoses, the reader is cautioned that our enumeration of causalgia is an upper bound to the number of cases meeting specified clinical criteria.

For the purpose of demonstrating the feasibility of gaining epidemiological information from administrative inpatient record systems, a patient-registry was constructed from the relevant portions of the IMR and IPDS data files. In addition

to the change from IMR and IPDS, there was also a change in the basic identifier used by the Army during the period of time spanned by the Vietnam conflict. Transition from the use of Service Number (SN) to Social Security Number (SSN) occurred in 1969. The Beneficiary Identification and Records Location System (BIRLS) of the Veterans Administration was used to partially re-create the SN to SSN conversion as required for the patient registry construction. In this particular application, the use of the BIRLS introduced an additional uncertainty in the records linkage process. The BIRLS coding did not retain the alphabetic prefix of the SN and it did not distinguish between a leading blank (such as would occur in a SN without a prefix) from a leading zero (such as would occur with some SSN). Confusion of multiple patients for the same patient did occur as a result of this shortening of identifier.

Summary records were located for 108 individuals with diagnostic codes consistent with causalgia. Review of the medical records of these individuals is in progress and will be reported as a case-series from the Vietnam era. The rate of causalgia per peripheral or multiple nerve injury was 15/1000 (i.e., causalgia occurred in 1.5% of the peripheral nerve injuries). When subdivided by body location (Table 1), the rate was only half as great for injury to the upper extremity (10/1000) as it was for injury to the lower extremity (19/1000) or injury to multiple sites (21/1000). For the upper extremity, the rate decreased with distance from the spinal cord but that relationship does not hold for the lower extremity. Since a significant fraction of the cases were in the multiple site category, further analysis of these data may allocate individuals to specific categories resulting in changes to the above stated relationships.

The annual number of peripheral and multiple nerve injuries peaked in 1968 while the largest number of causalgias (30 individuals) occurred in 1969. The annual rate of causalgia per 1000 peripheral and multiple nerve injuries appears to have increased toward the end of 1964-1973 period although the high rate in the final two years involved very small numbers as seen in Table 2.

The average age of 23.4 years of the individuals with causalgia exceeded by more than one year the average age of 22.2 years for all peripheral and multiple nerve injuries. The age-specific causalgia rates per 1000 peripheral or multiple nerve injuries are presented in Table 3 and show a dramatic increase for the over-35 year age bracket.

2. Psychiatric Hospitalization of Women in the Army

Description

This is a four-year prospective study which will determine the extent to which psychiatric hospitalization and diagnosis differ between male and female soldiers during the course of their first term enlistment. A further comparison will be made of hospitalization outcome of soldiers receiving traditional basic training versus soldiers receiving sex-integrated basic training. As this study progresses, it is anticipated that it will generate hypotheses which can be tested in epidemiological field studies.

Progress

The study consists of two cohorts: (a) a group of approximately 2,000 recruits entering active duty at Fort Jackson in October 1976 and (b) a group of approximately 20,000 recruits entering active duty at all basic training posts during the month of June 1977. The October 1976 cohort is incompletely identified as of this writing because of problems encountered in locating MILPERCEN records. The June 1977 cohort has been identified provisionally, and Table 4 entitled "GAG (Group and Gender): Description of June 1977 Cohort upon EAD (Entry on Active Duty)" characterizes this group.

It is too early and the data are too provisional to reach firm conclusions from the study as of this writing. Technical reports will be written and submitted for publication as the study develops and unfolds.

3. The Perceived Social Supports Instrument

Description

Research carried out in the psychosocial aspects of psychiatric epidemiology during recent years has indicated that availability and quality of an individual's social support network serves a major function of determining the health outcome for that individual in the sequence of responses to both acute and chronic stress. Much of the literature of military psychiatry indicates, as well, that the face-to-face social supports provided by the soldier's primary group serve in a powerful manner to affect his ability to successfully cope with the multiple stress of deployment and the battlefield. The perceived social supports instrument represents an initial attempt to develop a

quantifiable instrument for assaying and inventorying the self-perceived quality and frequency of contact of an individual with his network of "significant others" - those who comprise his social support system.

Progress

Work has been carried out to show the reliability and construct validity of this psychometric instrument which operationally measures people's perceptions of their affectual satisfaction when they go with important "Recognition Needs" to "Significant Others" in their lives. The "Recognition Needs": to be listened to; to be understood; to be accepted the way you are; to be cared for; to be encouraged when you're down; to have people to help solve problems; and to have people around when things are tough. The "Significant Others": Acquaintances; Close Friends; Blood Relatives; Person-Closest-To.

The Long Form of the instrument presents the subject with each of 28 combinations of "Recognition Need and Significant Other" groups in the following format (see Table 5): "When you go with X Need to Y Significant Other Group, how do they make you feel? Please put a mark on this (200 mm) line." The line runs from "Feel Worst Possible" at the far left through the middle, "Feel About the Same," to the far right, "Feel Best Possible." The Short Form of the Instrument collapses on "Recognition Needs" and presents the subject with each of the 4 "Significant Other" combinations (see Table 6): "When you go with your X Needs to Y Significant Other Group, how do they make you feel? (Same Line)"

The N=50 Reliability Sample and N=100 Validation Sample were randomly drawn from the WRAIR/WRAMC telephone directory. They represent a mixed military and civilian population, heterogeneous for race, gender, age, marital status and education. The Criterion Variables for N=100 Sample were the General Well Being Scale (GWB) (3) and the Hopelessness Scale (HS) (2).

The Test-Retest Reliability of the Long and Short Forms is 0.96 and 0.91, respectively (30 minute interval). Internal Consistency is 0.93 and 0.69 respectively (Cronbach's Alpha). Item-total correlations for both forms are all significant with a P-value ≤ 0.0001 . For Frequency of Contact and Life-Events Change (4) the multiple partial correlation of the Short Form with GWB is 0.53 and with HS is -0.50. The Interform substitutability of the Short Form for the Long Form is 0.85. Six times

more variance of the Criterion Variables is accounted for by Perception of Social Supports than by Life-Events Changes.

Controlling, first, for all levels of Life-Event Change, and, second for all levels of perceived Frequency of Contact, in both instances, as the sample moves from low to medium to high perceived supports (Long and Short forms), GWB uniformly increases and HS uniformly decreases. The odds ratio of being a case given Low Perceived Supports, Low Perceived Frequency of Contact, and High Life-Events change is 25.2/1.0.

4. Career Outcome Study

Description

Urine positivity for drugs of abuse is assumed to be a risk factor for psychiatric and physical illnesses as well as behavioral dysfunctions and ineffective performance. This study was designed to assess the long range behavioral implications of urine positivity and its potency as a factor of selection for risk for health and behavioral problems and to evaluate the medical evaluation procedure following designation of an individual as urine positive. A cohort demonstrating this presumed risk factor and controls who entered the Army during 1972-73 was defined in order to prospectively study their individual medical and military careers. The cohorts consist of 1967 individuals with positive urines and 2432 negative urine controls. The rate of matching was slightly over 80% for each cohort.

Progress

Analysis by the members of this cohort for specific precursors of illness and dysfunctional behavior is continuing. There are no special results to be reported for this year.

5. Psychiatric Disease Rates

Description

In 1976, historical data on psychiatric disease rates were consolidated into a paper which was filed with Defense Documentation Center (1). This paper presented neuropsychiatric hospitalization rates for the US Army from 1915 (pre-World War I) through the first six months of 1973 (post-Vietnam). From 1942 through mid-1973, psychiatric hospitalization rates were studied by presenting comparable data from the US Navy and, beginning in 1951, for the US Air Force, as well.

Progress

During the current reporting period, the task has been to update information initiated in the original paper. Pertinent data from Health of the Army from July 1973 through December 1978 have been extracted and filed electronically for easy access. Efforts to obtain comparable data from Navy and Air Force publications/agencies are underway.

Data on some twenty diseases or other morbidity indicators have been recorded for the years 1973 through 1978 for active duty Army personnel. Five catchment areas are studied: worldwide, continental United States, overseas, Europe, and western Pacific. In 1978, the hospitalization rate data are broken out by sex of the soldier.

Table 7 is a plot of psychiatric disease rates from 1915 through 1978; it utilizes data from the original paper and from the update. Table 8 summarizes morbidity differences according to the sex of the soldier for CY 1978.

Caution must be attached to interpretation of changes in the data trend line as it moves from mid-1973 to end-1973 because the system for tabulating, recording, and reporting data in Health of the Army underwent a transition from manual methods to automated methods at this time. Some of the categories of disease do not include the same specific diseases before and after the mid-1973 transition.

6. Suicide and Mortality

Description

Suicide is a major cause of death among active duty Army personnel. Approximately two American soldiers die by their own hand each week. Only motor vehicle accidents cause a greater number of violent deaths of soldiers in peacetime.

In collaboration with the Office of the Psychiatry Consultant, DASG, the Division has been studying the epidemiology of suicide of US Army active duty personnel since 1975. Findings for the years 1975-76 were summarized in a previous WRAIR annual report and are now set forth in a published article by Datel and Johnson (1979).

Progress

Work underway during the reporting period has been devoted to replicating the 1975-76 study for calendar years 1977-1978. There has again been collaboration with the Office of the Psychiatry Consultant, DASG. At the time of this writing, acquisition of the data set is nearing completion. Still existing are some problems in suicide identification and still missing are important data elements in some of the cases. The total number of completed suicides for the two-year period will apparently fall somewhere over 200, slightly less than the previous two-year period studied.

The search for adequate information has brought the principal investigator in touch with a number of record-keeping systems: HSC's, IPDS, CID's Crime Records Division, TAGO's Casualty Information System, AFIP's autopsy registry, the National Personnel Records Center in St. Louis, and the individual line of duty investigation file. When the data set becomes as complete as possible, the data will be banked electronically so that description and analysis can proceed along the lines following in the previous study. A manuscript describing the findings will then be submitted for publication.

A major problem was discovered and documented (2) during the conduct of this research. Reference here is to the questionable reliability in the mortality count and suicide count in either the medical and/or the personnel data-keeping systems. Until such fundamental questions as how many, and which, soldiers died during a given time period, and from what cause, are resolved, the outlook for obtaining definitive morbidity and mortality estimates from large scale, computerized data bases in the Army remains cloudy.

TABLE 1

ICDA-8 codes	Peripheral/ Multiple Nerve Injury n (%)	Causalgia n (%)	Causalgia per 1000 Peripheral/ Multiple Nerve Injury
952 Upper Arm	985 (13.8)	14 (13.0)	14
953 Forearm	1751 (24.5)	15 (13.9)	9
954 Wrist/hand	778 (10.9)	6 (5.6)	8
<hr/>			
952/954 Upper	3514 (49.2)	35 (32.4)	10
955 Thigh	814 (11.4)	23 (21.3)	28
956 Lower leg	1147 (16.1)	14 (13.0)	12
957 Ankle/foot	183 (2.6)	5 (4.6)	27
<hr/>			
955/957 Lower	2144 (30.0)	42 (38.9)	19
959 Multiple	1480 (20.7)	31 (28.7)	21
<hr/>			
952/957,959			
Peripheral			
Nerve Injury	7138 100%	108 100%	15

Table 1: Body Location of Peripheral/Multiple Nerve Injury and Causalgia in US Army, Vietnam, 1964-1973, first occurrence per individual. See text for diagnostic criterion.

TABLE 2

Time Period	Peripheral/ Multiple Nerve Injury		Causalgia		Causalgia per 1000 Peripheral/ Multiple Nerve Injury
	n	(%)	n	(%)	
1964	12	(0.2)	0	(0.0)	0
1965	154	(2.2)	0	(0.0)	0
1966	580	(8.1)	10	(9.3)	0
1967	1167	(16.4)	8	(7.4)	7
1968	2154	(30.2)	26	(24.1)	12
1969	1738	(24.4)	30	(27.8)	17
1970	879	(12.3)	21	(19.4)	24
1971	347	(4.9)	7	(6.5)	20
1972	73	(1.0)	4	(3.7)	55
1973	34	(0.5)	2	(1.9)	59
1964/1973	7138	100%	108	100%	15

Table 2: Annual Peripheral/Multiple Nerve Injury and Causalgia in US Army, Vietnam, 1964-1973, first occurrence per individual. See text for diagnostic criterion.

TABLE 3

Age	Peripheral/ Multiple Nerve Injury		Causalgia		Causalgia per 1000 Peripheral/ Multiple Nerve Injury
	n	(%)	n	(%)	
17-19	995	(13.9)	10	(9.3)	10
20-24	4967	(69.6)	74	(68.5)	15
25-29	736	(10.3)	11	(10.2)	15
30-34	224	(3.2)	3	(2.8)	13
35+	212	(3.0)	10	(9.3)	47
UNK	3	(0.0)	0	(0.0)	0
Total	7138	100%	108	100%	15

Table 3: Age-specific Peripheral/Multiple Nerve Injuries and Causalgia in US Army, Vietnam, 1964-1973, first occurrence per individual. See text for diagnostic criterion.

TABLE 4

GAG: Description of June 1977 Cohort Upon EAD

N	<u>TOTAL</u>		<u>MEN</u>		<u>WOMEN</u>	
	n	(%)	n	(%)	n	(%)
N by Post						
BLISS	1,029	5.6	1,029	6.0	--	--
DIX	3,533	19.1	3,533	20.5	--	--
JACKSON	5,272	28.5	4,782	27.8	490	38.5
KNOX	2,309	12.5	2,309	13.4	--	--
WOOD	3,658	19.8	3,658	21.2	--	--
McCLIN	1,030	5.6	247	1.4	783	61.5
SILL	1,667	9.0	1,667	9.7	--	--
AGE (mean)	19.4		19.3		20.3	
MARITAL: single	<u>16,878</u>	92.1	<u>15,802</u>	92.7	<u>1,076</u>	84.5
	<u>18,318</u>		<u>17,045</u>		<u>1,273</u>	
RACE: White	11,933	66.1	10,950	65.2	983	78.3
Black	5,513	30.6	5,276	31.4	237	18.9
Other	<u>597</u>	<u>3.3</u>	<u>561</u>	<u>3.3</u>	<u>36</u>	<u>2.9</u>
	<u>18,043</u>	<u>100.0</u>	<u>16,787</u>	<u>99.9</u>	<u>1,256</u>	<u>100.1</u>
EDUC: < HS	6,514	35.3	6,362	37.0	152	11.9
= HS	<u>11,953</u>	<u>64.7</u>	<u>10,832</u>	<u>63.0</u>	<u>1,121</u>	<u>88.1</u>
	<u>18,467</u>	<u>100.0</u>	<u>17,194</u>	<u>100.0</u>	<u>1,273</u>	<u>100.0</u>

TABLE 4 (Cont'd)

GAG: Description of June 1977 Cohort Upon EAD

INTELL:					
AGQT (M-SD)	50.7 - 19.7	49.0 - 19.0	74.2 - 13.1		
GT (M-SD)	102.6 - 15.2	101.6 - 15.1	115.9 - 10.4		
RELIGION					
NO PREF	5,042	4,716	27.4	326	25.6
FUND PROT	1,163	1,074	6.2	89	7.0
TRAD PROT	2,644	2,382	13.8	262	20.6
BAPTIST	4,648	4,408	25.6	240	18.9
ROM CATH	3,969	3,671	21.3	298	23.4
JEWISH	60	52	0.3	8	0.6
OTHER/Unk	972	922	5.3	50	3.9
ENLISTMENT:					
FIRST	17,584	16,341	97.5	1,243	99.4
SECOND	425	418	2.5	7	0.6
	18,009	16,759	100.0%	1,250	100.0%

TABLE 4 (Cont'd)
GAG: Description of June 1977 Cohort Upon EAD

	<u>TOTAL</u>		<u>MEN</u>		<u>WOMEN</u>	
	n	(%)	n	(%)	n	(%)
COMPONENT:						
REGULAR	18,325	99.1	17,052	99.0	1,273	100.0
NATL GUARD	10	0.1	10	0.1	--	--
RESERVE	163	0.9	163	0.9	--	--
TERM OF SERVICE:						
1 YR	1	0.0	1	0.0	--	--
2 YR	113	0.6	113	0.7	--	--
3 YR	12,556	67.9	11,662	67.7	894	70.2
4 YR	5,752	31.1	5,373	31.2	379	29.8
5 YR	1	0.0	1	0.0	--	--
6 YR	7	0.0	7	0.0	--	--
CITIZENSHIP:						
NATIVE	18,122	98.0	16,873	98.0	1,249	98.1
NATURLZD	31	0.2	27	0.2	4	0.3
ALIEN	313	1.7	296	1.7	17	1.3
DERIV	32	0.2	29	0.2	3	0.2
HOME OF RECORD:						
California	1,395	7.5	1,276	7.4	119	9.3
New York	1,138	6.2	1,063	6.2	75	5.9
Ohio	1,108	6.0	1,032	6.0	76	6.0
Texas	1,102	6.0	1,024	5.9	78	6.1
Illinois	916	5.0	851	4.9	65	5.1
Pennsylvania	891	4.8	819	4.8	72	5.7
Florida	853	4.6	820	4.8	33	2.6

TABLE 4 (Cont'd)

GAG: Description of June 1977 Cohort Upon EAD

Michigan	633	3.4	571	3.3	62	4.9
North Carolina	582	3.1	566	3.3	16	1.3
Georgia	536	2.9	518	3.0	18	1.4
Missouri	510	2.8	478	2.8	32	2.5
Other	8,834	47.8	8,207	47.6	627	49.3

TABLE 5

SUBJECTIVE INDEX OF SOCIAL SUPPORT SATISFACTION₂₈ (SISSS₂₈)

- (1) to be listened to (heard)
 (2) be understood
 (3) to accepted the way you are
 (4) be encouraged (given a "shot in the arm") when you're down
 (5) be cared about
 (6) rely upon people when things are tough
 (7) have people around to help solve problems
- (a) your acquaintances (people at work, school, or in the neighborhood)
 (b) your close friends
 (c) your blood relatives
 (d) the person you're closest to

When you need to:

And you go to:

How do(es)
 they (he/she):

make you feel?

Please place a mark on the following line:

FEEL WORST POSITIVE	FEEL NO DIFFERENT (ABOUT THE SAME)	FEEL BEST POSITIVE
------------------------	--	-----------------------

(Each of the 7 Recognition Needs will be interacted with each of the 4 Significant-Other categories, so that 28 different combinations, each on a separate piece of paper, will be

TABLE 5 (Cont'd)

SUBJECTIVE INDEX OF SOCIAL SUPPORT SATISFACTION₂₈ (SISS₂₈)

presented to the individual. E.G., "When you need to be listened to and you go to your acquaintances, how do they make you feel?"

TABLE 6

When you go with your:

How do(es)

Please place a mark on the following line:

FEEL WORST
POSSIBLE

(Each of the 4 Significant-Other categories will be interacted with the collapsed Recognition-Need category, "Needs," so that 4 different combinations, each on a separate piece of paper, will be presented to the individual. E.G., "When you go with your needs to your acquaintances, how do they make you feel?")

TABLE 7
 PSYCHIATRIC DISORDER IN U.S. ARMY (ACTIVE DUTY, WORLDWIDE)
 FROM PRE-WORLD WAR I THROUGH 1978
 * = NEUROPSYCHIATRIC DISORDER
 X = PSYCHIATRIC DISORDER, INCLUDING DRUGS AND ALCOHOL
 O = PSYCHIATRIC DISORDER, NOT INCLUDING DRUGS AND ALCOHOL

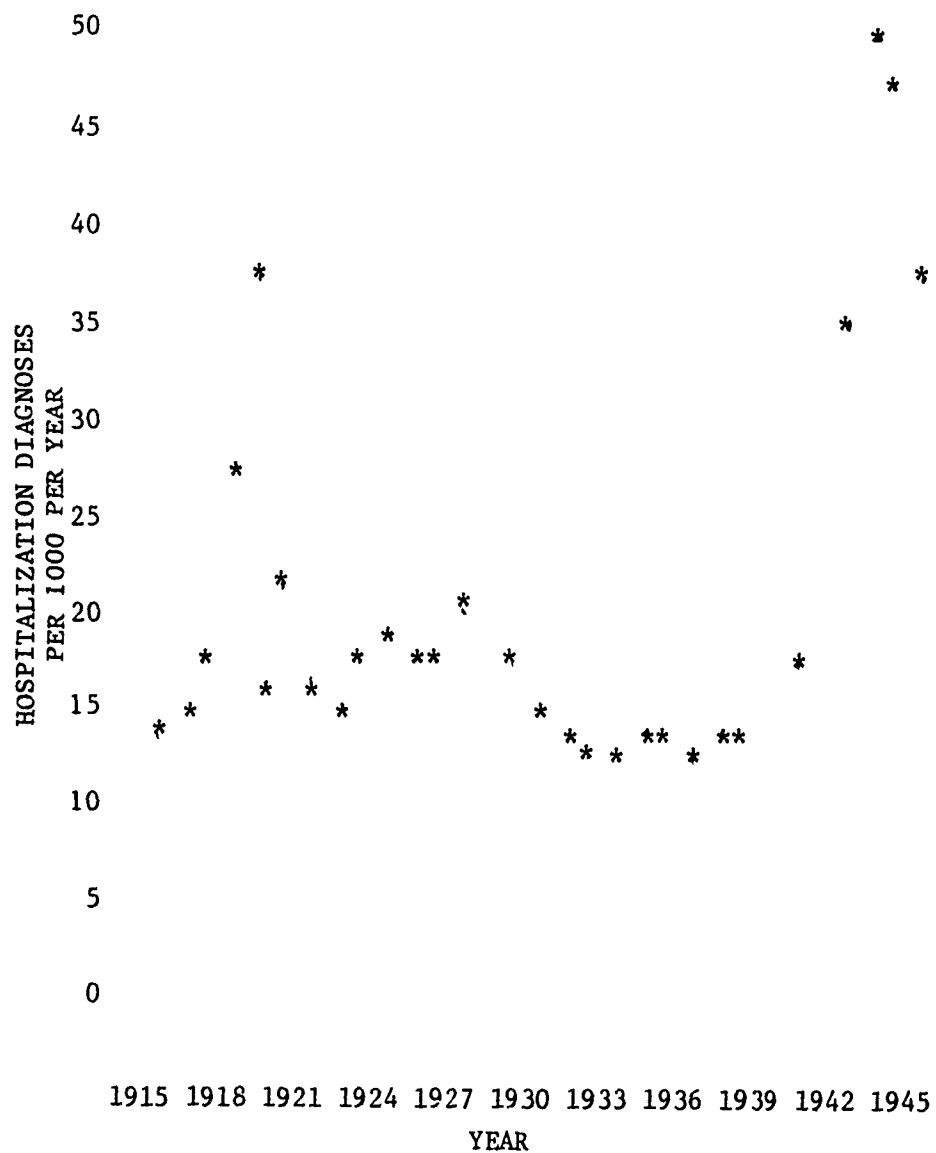


TABLE 7 (Cont'd)
 PSYCHIATRIC DISORDER IN U.S. ARMY (ACTIVE DUTY, WORLDWIDE)
 FROM PRE-WORLD WAR I THROUGH 1978
 * = NEUROPSYCHIATRIC DISORDER
 X = PSYCHIATRIC DISORDER, INCLUDING DRUGS AND ALCOHOL
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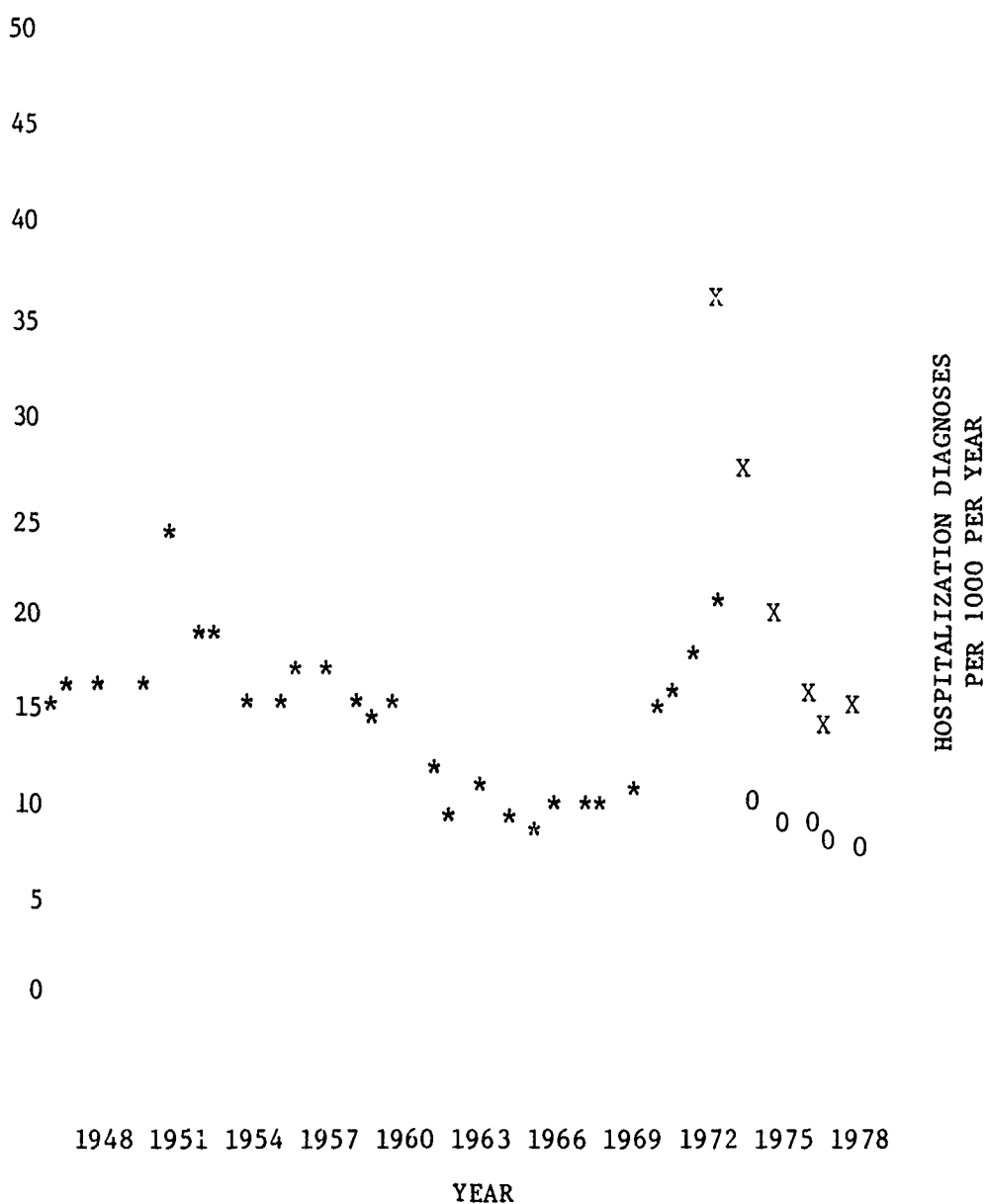


TABLE 8
COMPARISON OF U.S. MALE SOLDIERS WITH U.S. FEMALE SOLDIERS WORLDWIDE ON SELECTED DISEASES/
INDICATORS FOR CALENDAR YEAR 1978 (SOURCE: Health of the Army)

Disease/Indicator	ICDA-8	Rate (hosp episodes per 1000 per year)		Rate Ratio Women:Men
		Men	Women	
Psychiatric	290-301,307	8.12	18.51	2.3:1
Drugs/alcohol	303,304,7932, 793A-793M	7.53	5.15	.7:1
Infectious intestinal	004,006-009	1.69	6.35	3.8:1
Hepatitis	070,5730,9992	2.82	2.12	.8:1
Upper respiratory infection	460-474	19.33	31.49	1.6:1
Genital	600-607,612-629	3.88	34.63	8.9:1
Fractures	800-829	7.31	4.61	.6:1

TABLE 8 (Cont'd)

COMPARISON OF U.S. MALE SOLDIERS WITH U.S. FEMALE SOLDIERS WORLDWIDE ON SELECTED DISEASES/
INDICATORS FOR CALENDAR YEAR 1978 (SOURCE: Health of the Army)

Disease/Indicator	ICDA-8	Rate (hosp episodes per 1000 per year)		Rate Ratio Women:Men
		Men	Women	
Adverse effects, chemical subst	960-989	2.02	6.92	3.4:1
All diseases	000-799	92.40	263.48	2.9:1
All injuries	800-999	20.06	21.17	1.1:1
On hospital rolls		4.46*	9.82*	2.2:1
Occupying hosp beds		.27**	.44**	1.6:1

* Average daily number of soldiers per 1000 strength

** Average daily percentage of strength

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1. Dattel, W. E. & Johnson, A. W., Jr. "Suicide in United States Army Personnel, 1975-1976." Military Medicine, 144:239-244, 1979.
2. Dattel, W. E. "The Reliability of Mortality Count and Suicide Count in the United States Army." Military Medicine, 144:509-512, 1979.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION*	2 DATE OF SUMMARY*	REPORT CONTROL SYMBOL DD-DRAC(AR)636	
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY*	6 WORK SECURITY*	7 REGRADING*	8A DISSEM INSTR*	8B SPECIFIC DATA CONTRACTOR ACCESS	9 LEVEL OF SUM
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10 NO /CODES*	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
A. PRIMARY	62771A	3E/62771A804		00		048	
B. CONTRIBUTING							
C. CONTRIBUTING	CARDS 114F						
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(U) Military Stress: Circadian and Ultradian Factors							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS*							
016200 Stress Physiology 013400 Psychology							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
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17 CONTRACT/GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA				PRECEDING		B. FUNDS (In thousands)	
B. NUMBER*				FISCAL YEAR		79	
C. TYPE				CURRENT		5.0	
D. KIND OF AWARD				80		537	
E. AMOUNT:							
F. CUM. AMT.							
20 RESPONSIBLE DOD ORGANIZATION				21 PERFORMING ORGANIZATION			
NAME* Walter Reed Army Institute of Research				NAME* Walter Reed Army Institute of Research			
ADDRESS* Washington, DC 20012				ADDRESS* Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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				SOCIAL SECURITY ACCOUNT NUMBER			
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				NAME: Thorne, D. Ph.D.			
23 KEYWORDS (Precede EACH with Security Classification Code) (U) Stress; (U) Biological Rhythms; (U) Chronobiology; (U) Electrophysiology; (U) Performance; (U) Psychophysiology; (U) Human Volunteer							
24 TECHNICAL OBJECTIVE, 25 APPROACH, 26 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code)							
<p>23. (U) Achievement of an understanding of the temporal organization of biological functions attendant upon sustained exposure to stressors in military environments. Information developed provides indicators of the magnitude and time-course of stressor induced behavioral and physiological disorders that are the precursors of the production of psychiatric and combat casualties.</p> <p>24. (U) Monitoring techniques are employed in the laboratory and in the field to obtain detailed behavioral, electrophysiological, and biochemical measures of functioning during sustained operations. A variety of time series analysis techniques are applied to these data to assess changes that precede and accompany stress responses.</p> <p>25. (U) 78 10 - 79 09 Two studies of time zone translocation (Jet Lag) were completed, involving 300 troops deploying to Europe. Various countermeasures against dyschronism and serial cognitive performance measures were experimentally employed. Preliminary analysis of data indicates expected decrements and gradual recovery on several tasks. Countermeasures reduced subjective fatigue, but did not selectively affect performance. Older subjects appear to respond more to countermeasures, and had more positive self-ratings of performance than younger subjects, though age effects were not entirely consistent. Laboratory studies are planned to clarify the several issues raised by these field studies. Civilian firefighters and fire/rescue dispatchers were studied in relation to stress of occupational life threat and shift work. Analysis is in initial phase. Studies of rhythmic aspects of affect, activation, cognitive performance, verbal perception, and laterality of cerebral hemispheric function are underway or in preparation. For technical report see Walter Reed Army Institute of Research Annual Progress Report, OCT 78-30 SEP 79.</p>							

Project 3E/62771A804 MILITARY PSYCHIATRY

Work Unit 048 Military Stress: Circadian and Ultradian Factors

Investigators.

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Description

The organization of physiologic function and performance in military environments are studied in the temporal domain. The magnitude and time-course of the deleterious effects of stressors, and of progressive adaptation processes, are investigated in the context of stress exposures, such as sleep deprivation, continuous combat operations, temporal dysynchronization, shift work, and life threat, which are precursors of performance decrements and casualty losses. Upon a technological base developed within or supported by the Department, non-intrusive, field portable sensors and recorders of biological and behavioral parameters are applied in naturalistic settings in order to define in realistic terms the functional topography and costs of stressful events. Field experience in turn leads to laboratory testing of select hypotheses concerning primary stress phenomena and potential interventions or countermeasures. In continuum, the technical base is broadened, and methods of analysis are refined.

The extent and scope of certain studies described represent organizational efforts involving the collaboration, thoughtful contributions, and resources of many agencies, departments, and individuals. Personnel of HQ, Division of Neuropsychiatry and the Departments of Psychiatry and Neurosciences were involved as co-investigators and research assistants in the "Jet Lag" studies. Special efforts in central and field support were afforded by HQ, USAMRDC, HQ, WRAIR, Division of Logistics, WRAIR, and the U.S. Army Medical Research Unit, Europe.

Progress

1. Studies of Time-Zone Translocation ("Jet Lag")

Introduction. Disruption of the circadian system is widely

recognized as underlying the general malaise, fatigue, and psychological deficits which most travelers experience following rapid transmeridial flight. Previous attempts at developing chronobiotics to hasten adaptation to new time zones have met with limited success (Christie and Moore-Robinson, 1970; Simpson, Bellamy, Bohlen, and Halberg, 1973). Recently, Ehret and his colleagues (Ehret, Groh, and Meinert, 1978) have suggested a series of nonpharmaceutical chronobiologic measures designed to accelerate phase shifting of the mammalian circadian system. While laboratory experiments have confirmed the efficacy of these manipulations for shifting the body temperature rhythm in animals (Ehret, Potter, and Dobra, 1975; Ehret et al., 1978), no evidence is yet available concerning their effectiveness in humans, especially in those undergoing rapid transmeridial flight.

The purpose of present studies was to provide such data in the context of a military deployment. Countermeasures were designed partially on the basis of Ehret's model but tailored to the operational requirements of a large-scale military exercise. The latter prohibited the use of any countermeasures before the day of departure and restricted them to those which could be carried out without disruption of mission accomplishment. The overall strategy was to induce a more rapid phase advance of the circadian system by controlling the timing of the following factors: rest-activity schedules, social interaction, caffeine/theophylline consumption, and meals. To enhance the impact of altered mealtiming, subjects underwent a moderate fast on the day of departure and were permitted only a limited intake of carbohydrates throughout that day. Also, dimenhydrinate was administered to facilitate a rapid advance in subjects' rest-activity schedule on the aircraft. Whenever possible, psychological time cues were altered to reflect destination time.

These abbreviated reports are based on preliminary analyses of data and, therefore, should not be construed as representing the final conclusions of the study. A more definitive statement about the findings will be published in the near future.

Jet Lag I. This study examined the effectiveness of such countermeasures by comparing two groups of male soldiers (age 18-38 years) from the 2nd Armored Division (Forward), airlifted from Texas to W. Germany. Oral temperatures were measured at 4-hr. intervals during four working days prior to departure. Every 4 hrs. throughout this 96-hr. span a subset of 15 subjects in each group maintained a circadian diary, had their temperatures taken, performed two simple behavioral tasks, and completed a subjective fatigue checklist. Countermeasures instituted on the morning of departure advanced the timing (+6 hrs.) of

meals, rest/activity, and social interaction. The experimental subjects (N=84) ate lightly and avoided caffeine on the day of departure, took 100 mg of dimenhydrinate before lights out at 1700 CDT, and ate a large high protein breakfast with coffee before landing at 0600 CET. The control subjects (N=95) underwent usual airlift procedures on a separate flight. Upon arrival all subjects were studied every 4 hrs. for 6 days. The countermeasures significantly reduced sleeping and subjective fatigue during the first two days (Figs. 1 & 2). Single digit addition was unaffected for both groups. The mean oral temperatures of both groups manifested appropriate circadian phase shifts within 24-48 hrs. (Fig. 3); however, the countermeasures produced a more rapid phase adjustment and recovery of amplitude for subjects \geq 30 years old (Fig. 4). The use of chronobiologic countermeasures, even if limited to the day of departure, may help overcome the effects of transmeridial flight more rapidly.

Jet Lag II. The second study attempted to replicate and extend the previous findings that chronobiologic countermeasures hasten circadian adjustment following rapid transmeridial flight. The experiment comprised two parts, both conducted during a winter field deployment exercise (REFORGER 79) of the 1st INF DIV. from Ft. Riley, Kansas to West Germany (+7 hours). Subjects in both parts were administered morning, noon, and evening tests for four days in Kansas, and for 3-5 days in Germany with the inclusion of additional night tests. In Part I, two groups of 60 subjects each maintained sleep logs, completed self-confidence scales and a fatigue checklist, and performed an encoding-decoding task at each session. The experimental group underwent the countermeasures as described previously, while controls followed normal military airlift procedures. Part II compared a group of 31 young subjects (mean age 21.0 years) with 29 older subjects (mean age 34.2 years) following deployment without any countermeasures. They completed an extensive cognitive test battery at each session in addition to the test in Part I. Despite arduous field conditions the countermeasures again reduced the amount of subjective fatigue for 48 hours following arrival (Fig. 5). Encoding-decoding performance diminished in both Part I groups after deployment but was not differentially affected by the countermeasures. Performance on most Part II cognitive tasks deteriorated following arrival and then gradually recovered, depending on the task, over 24 to 72 hours (Fig. 6). Some suggestion of enhanced diurnal variations emerged from these data, but there were no consistent age effects. However, self-ratings of performance on these tasks indicated that the younger subjects consistently underestimated their cognitive abilities after the first day in Germany. The only other age difference was that the older group slept less in both Kansas and Germany.

Laboratory Investigation. Followup laboratory research is currently being undertaken to confirm and extend these findings from the field. The laboratory's isolation chamber has been structurally modified to include toilet, bath, kitchen, and sleeping facilities so that up to four individuals can live within its confines for 3 to 4 weeks. A protocol is under review, which when implemented will investigate temporal factors of cognitive performance using a more complete cognitive test battery.

2. Studies of Affective Responses to Continuous Performance

Fire Direction Center Study. Continuing analysis of data from the Fire Direction Center Study (FDC) has revealed clear evidence of disturbance in the balance between positive and negative affect under continuous performance - sleep deprivation conditions, with individual changes in the amplitude of periodicities of affective behavior.

In this study two scales, Zuckerman's Multiple Affect Adjective Check List and the Activation-Deactivation Adjective Check List were blended in five randomized forms of a mood-activation scale. Each man in the FDC teams filled out one of these in four equally spaced breaks per 24-hour period. Separate scores for anxiety, hostility and depression were converted to Z scores, after normal distributions were demonstrated. Several points rapidly became clear: (1) it seems superfluous to differentiate among anxiety, hostility and depression since all three affects changed concurrently in the same direction with similar orders of magnitude; (2) it seems parsimonious to postulate paradigmatic biases affecting the processes that govern affective balance in favor of negative affective peaks; (3) periodicities were not necessarily eliminated and may in individual cases have been exaggerated, although the 4 per day sampling rate limited resolution; (4) the replicated 48-hour runs indicate that individuals are likely to react similarly to repetition of the experience; and (5) a consistent peak of negative affect near the end of the runs were evident, but this was accompanied by a return to beginning of baseline following the recovery period.

Consistent with the overall increases in negative affect, performance decrements focused on those tasks least constrained by time demands, and coincided with overly self-critical behavior. Analogous to clinical depression, at least two phenomena appeared: an actual performance deficit and an unrealistically perceived inadequacy of performance. Although future studies should specifically address this issue, at least three factors of the sleep-deprivation/continuous performance situation probably interact to

impair performance: (1) sleep deprivation with its accompanying subjective sense of fatigue and deactivation (the latter being measurable by the activation part of the mood activation scale); (2) increasing inability to regulate the positive-negative affective balance of amplitude and rhythmicity; and (3) cognitive deficits that involve such functions as memory and logical reasoning.

The analysis of these data is proceeding with collaboration with the Department of Psychiatry.

Rhythmic Aspects of Cognitive Performance, Affect, and Motor Activity. A protocol is under review which capitalizes upon the above findings, and examines the relationships among rhythms in cognitive performance, affect and subjective activation or arousal, and patterns of spontaneous hand or arm motor activity. These phenomena are viewed as co-ordinated manifestations of the basic rest/activity cycle, that should jointly correlate with the efficient performance of military tasks. Development of a transducer for detecting fine-motor hand activity has begun, using bistable magnetic wires (Wygand effect). Hand activity may be isolated from general body activity, as determined by Actigraph transducers. In concert with progressive development of a Performance Assessment Battery, selected tests of cognitive function and performance have been transformed to software automation.

Rhythmic Structure of Verbal Perception. The aforementioned protocol has been extended to a study exploring rhythms and hemispheric differentiations in the perception and identification of verbal stimuli, visually presented. Preliminary planning has been completed in collaboration with Dr. Harvey Babkoff, Senior National Science Foundation Research Fellow. Development of instrumentation and automation of tasks is under way.

Electroencephalographic Activity During Performance Task Studies. An electroencephalographic (EEG) record collected during the course of pilot work involving a speed-accuracy tradeoff task is being digitized for analysis in collaboration with Dr. Will Gersch, Department Computer Sciences, University of Hawaii. It is hypothesized that alterations in affect, cognitive functioning, consciousness and motor and sensory functioning experienced by the subject will be associated with predictable changes in the differential patterns of hemispheric function. The methodology to be used in the analysis (a realization of Kellbach's minimum discrimination information in the time and frequency domain) should reveal whether EEG differences within or between hemispheres contain enough information about cognitive and

affective state changes to consider further development of the EEG as a dependent variable in indexing affective and cognitive biorhythms.

Affect and Social Support Factors. In collaboration with Dr. Robert Blaik, Department of Psychiatry, a supplementary analysis of his study of quantification of perception of social support is proceeding. Dr. Blaik's data (see Annual Report, Dept. of Psychiatry) is being viewed in terms of: (1) the relative overall amounts of positive and negative affect associated with fixed categories of relationships; (2) a theoretic measure (\bar{H}) of unevenness in the distribution of affect among relationships; and (3) relative effects of people, needs, and people-need interaction on the allocation of affect to relationships. Preliminary results indicate: (1) independently significant effects of overall positive and negative affect on a criterion combining a General Well Being and a Hopelessness Scale and (2) in positive correlation between \bar{H} and overall amount of positive affect. Further exploration and extension of these observations is planned. A questionnaire is being devised which surveys relevant life areas and delineates relationships with ongoing mood-activation measures.

3. Firefighters Study

Background. In peacetime, certain civilian occupations may provide a close parallel to combat stress via the actual and immediate presence of life threat and unforgiving demands on human capacities to take action. A pilot study was begun this year to assess the influence of these natural stressors on the structure of behavior, social organization, and physiologic function, and to develop and refine technological capabilities for making such assessments. A cooperative agreement with the Montgomery County Fire and Rescue Services yielded an opportunity to study individual volunteer subjects for whom emergent action and life threat (direct or indirect) are occupational realities. Two separate cohorts of volunteers became involved; 1) over twenty fire and rescue professionals involved in on-the-scene operations, and 2) thirteen communications personnel at the Emergency Operations Center (EOC) in Rockville, Maryland, who receive and process all incoming emergency calls within the county and who coordinate and dispatch equipment and manpower from the field. For several reasons initial phases of study focused on the latter group. Dispatchers worked in stable social/work triads, working regular shift schedules with a continuous albeit modulated work load. They were readily monitored by existing techniques. The function of the EOC within the county is analogous to that of Tactical Operations Centers (TOC) in modern combat and is of major interest to those who investigate the impact of subtle stressors on higher order mental work (e.g., decision making).

Hence, given the limited available research manpower, a systematic study could be devised with potentially high density payoff in terms of regularized data collection, meaningful if tentative conclusions, and increased precision of analytical approaches. Several work periods and emergency responses were observed in the field providing a background understanding of fire and rescue operations, jargon, and social milieu. It became clear that the infrequency of major incidents, the routine nature of most calls, the extended periods of lax time invested to study a single major event, and the non-systematic means available for describing events precluded an unlimited commitment of investigative resources for the present. The EOC study and samplings of field experience yielded ample opportunity to sharpen the methodology and to provide a basis for planning efficient approaches to the problems presented by the field environment. The influence of direct life threat on physiology and adaptive behavior remains the nuclear issue and the analysis of rare, transient and pulsatile exposure to stressors remains a major challenge to current research methods.

Procedures. Evaluation of subjects and events were pursued within three general categories of assessment: 1) health status and professional/social/personality traits (an ipsative data base), 2) monitoring of rest/activity patterns and heart rate for periods extending across work, offtime, sleep, and shift-scheduled transitions, and 3) recording of work period events hypothesized to drive or entrain the observed patterns of physiologic behavior. For the first category medical record review, verbal history, and physical examinations were obtained along with a series of questionnaires concerning personality, professional and social attitudes, and autonomic perceptions. For the second category the Actigraph was employed and electrocardiogram (ECG) monitoring was obtained for 32-48 hours of the first and last segments of each shift. The events within work periods were monitored by participatory observation, by voice recording of all radio/telephone traffic, and, in the case of the EOC, by quantitative recording of traffic load in 5-minute epochs. Continuous recording of activity and intermittent samplings of ECG and work load were thus obtained. Heart rate variability (variance within epoch) was also computed from ECG data and similarly arrayed. Figure 7 is an example of the raw data acquired. During all work periods involving ECG monitoring, subjects were under direct observation, notations were taken on position and activity, magnitude of work load, and subjectively assessed social interaction.

Additional studies of subjects' health and responsivities are in preparation. They include blood chemistries, responses to maximal treadmill exercise, and assessment of cardiovascular reactivity to minimal stress.

Initial Results. The exploitation of the massive data archives as acquired in the EOC requires meticulous exploration by both existing and innovative analytic techniques. Global interactions, i.e. weakly positive but significant correlations, have been demonstrated among activity data, heart rate, heart rate variability, and quantity of radio/telephone traffic or work load. In several cases, local but sustained negative correlations exist between work load and cardiac variability. Of particular interest are the occasional negative spikes in heart rate variance (see Fig. 8). These physiological events occur in no clear relationship to either workload or activity. However, they may point to particularly stressful work load periods, a hypothesis suggested by the literature (e.g. Sayers, 1971). The examination of verbal and emotional content of those voice recordings concomitant with these and other patterned physiological events is planned.

Circadian variations of activity and heart rate, their adaptation to shift changes, and event-induced disruptions of rest are readily apparent in the raw data. These variations are more precisely organized by time-series analysis using the method of multiple complex demodulation developed in this Department. In this way phasic adjustments in circadian periods and amplitude to shift-work schedules are displayed as in Fig. 9. Analysis of relative energy and of energy transitions in circadian and ultradian components is under way with the focus on shift-transitions with exploration for entrainment phenomena related to quantity or content of workload. An additional physiologic measure will become available with development of the means for spectral and microtremor analysis of voice recordings when such analysis is combined with verbal and situational content analysis.

The major challenge of the immediate future is to automate and accelerate explorational statistical and analytical procedures. This development will be applicable to the several other sources of data available to this department. The result of such explorations will be the synthesis of testable hypotheses, new laboratory research designs, and more efficient field research.

COUNTERMEASURES REDUCE POST-FLIGHT SLEEP

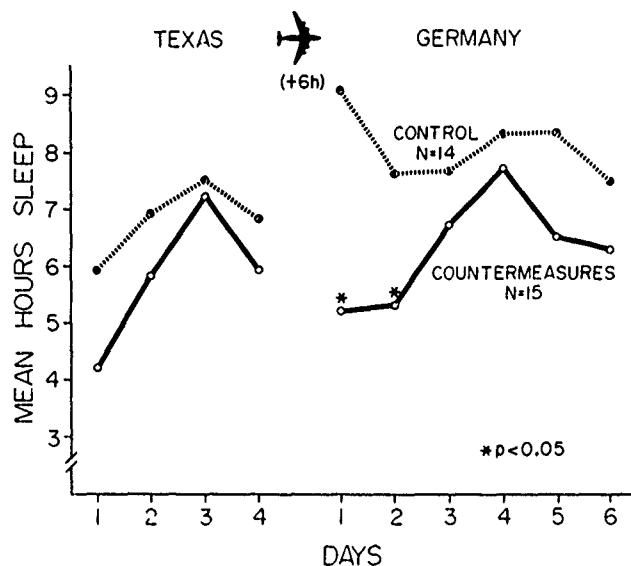


Fig. 1 - Sleep, before and after deployment.

EFFECTS OF COUNTERMEASURES ON POST-FLIGHT FATIGUE

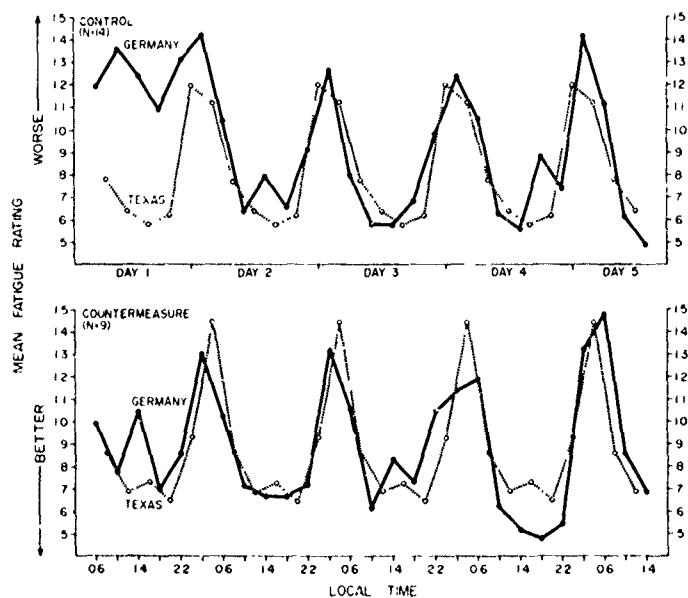


Fig. 2 - Self-reported fatigue ratings after deployment, compared to Texas baseline.

GROUP BODY TEMPERATURE ADJUSTMENT AFTER FLIGHT (+6h)

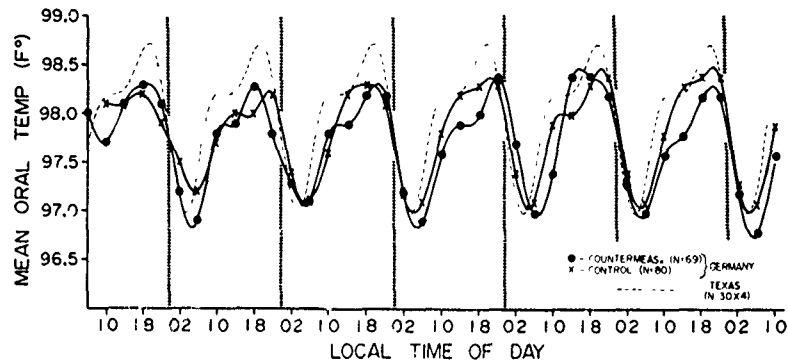


Fig. 3 - Post-flight body temperatures (large groups); spline-fit curves compared to that for pooled Texas baseline groups.

POST-FLIGHT ADJUSTMENT OF BODY TEMPERATURE IN OLDER SOLDIERS (≥ 30 yrs.)

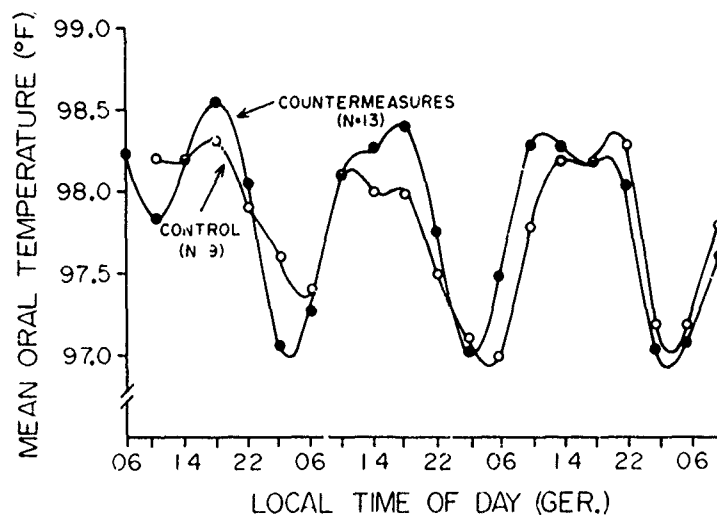


Fig. 4 - Post-flight adjustment of temperature in older soldiers.

COUNTERMEASURES REDUCE POST-FLIGHT SUBJECTIVE FATIGUE RATINGS

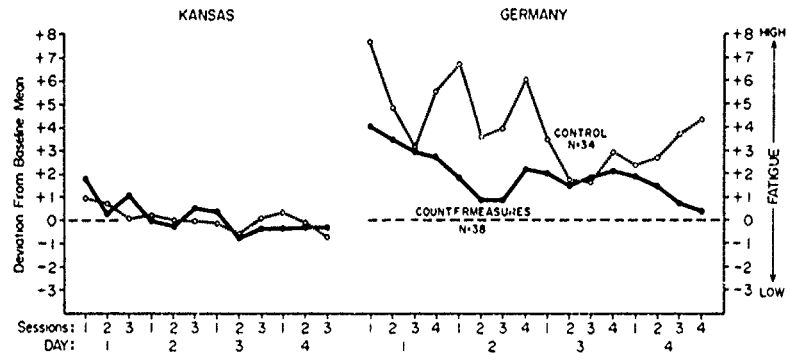


Fig. 5 - Effect of countermeasures on self-rated fatigue.

EFFECTS OF TRANSMERIDIAL FLIGHT ON LOGICAL REASONING BY OLDER AND YOUNGER SOLDIERS

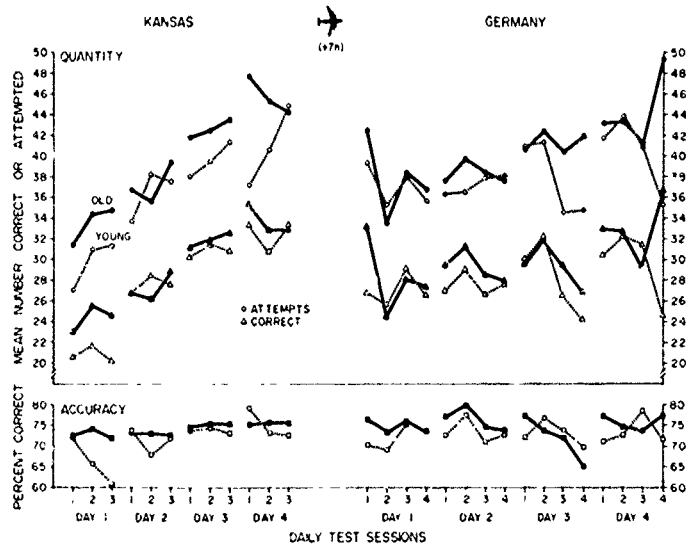


Fig. 6 - Logical Reasoning Task; performance of older and younger soldiers before and after deployment.

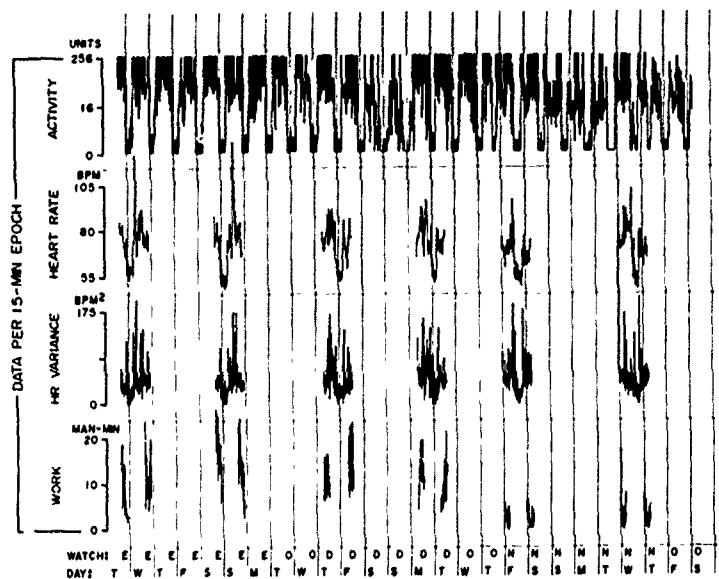


Fig. 7 - Activity, workload, and heart rate data for 26 days in shiftworker (EOC).

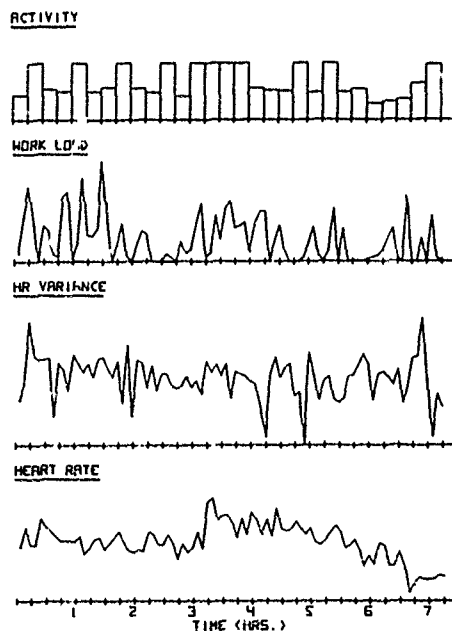


Fig. 8 - Activity, workload, and cardiac measures acquired during a single EOC watch (evening shift).

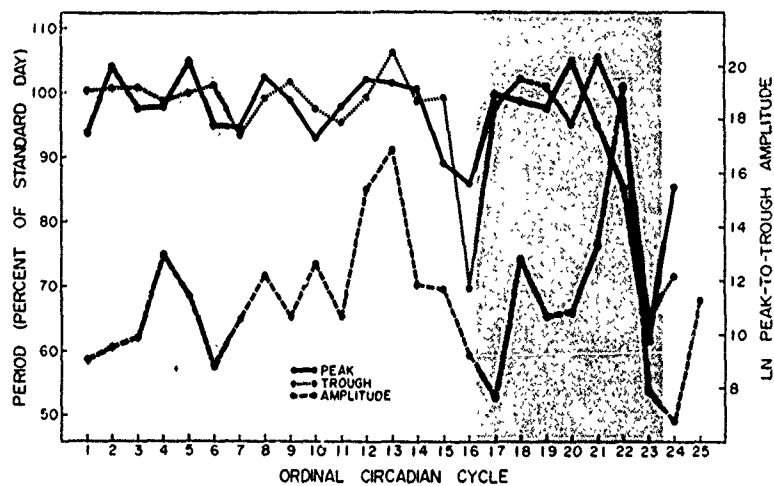


Fig. 9 - Circadian periods and amplitudes derived from complex demodulation analysis of activity data in Fig. 7.

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Project 3E162771A805

MICROWAVE INJURY

Project 3E/62T11A804 MILITARY PSYCHIATRY

Work Unit 048 Military Stress: Circadian and Ultradian Factors

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL DD FORM 1498 (AR) 636	
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10. NO / CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
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24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Publish individual paragraphs identified by number. Precede text of each with Security Classification Code)							
23. (U) To provide technical and medical information to the Surgeon General, system developers and agencies responsible for safety standards in order to protect the health and effectiveness of military units and affected civilian populations in microwave and RF environments. This requires analysis of the biophysics and bioeffects attributable to non-ionizing radiation under laboratory conditions which reasonably simulate and/or predict operational exposures.							
24. (U) To perform basic and applied research on the problem of microwave and RF interactions with biosystems at all levels of analysis from the cellular and molecular to metazoan physiology, pathophysiology, and behavior. This requires development of measurement systems for dosimetric analysis, in vitro and in situ; the evaluation of frequency, power level, polarization and modulation as important parameters of the radiation; and the use of low level energy to assess the functional state of cells and tissues.							
25. (U) 78 10 - 79 09 Progress has included the demonstration of feasibility for non-invasive microwave dosimetry using scattering parameters and time delay spectroscopy; development of network analysis methods for high speed, broad band measurement of permittivity in biological tissues; development of methods for improved spatial resolution in microwave images of isolated organs; preliminary studies to assess functional states of cells and tissue by in situ permittivity analysis; development of microwave transparent electrodes for temperature measurement and induced electric field strength in situ; and development of high power pulse exposure facility for radar simulation. Conducted studies of thermal cataracts of the ocular lens. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.							

Project 3E162771A805 MICROWAVE INJURY

Work Unit 041 Biological Interactions with and Hazards of Microwave Radiation

Investigators.

Principal: LTC Lawrence E. Larsen, M.D., MC

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ADMINISTRATIVE ACTIVITIES

This report period was one of continuously expanding administrative demands. Major items were facility construction projects, staff reorganization, chairmanship of the Tri-Service Electromagnetic Radiation Panel (TERP), manpower studies and programmatic reviews at several levels outside of DA including hearings in the U.S. House of Representatives.

A. Construction

This report period marks the beginning of the third year of a construction project for renovation of the WRAIR microwave exposure facilities at Forest Glen. This project was originally estimated to take 6 to 9 months. The delays we have experienced were related chiefly to problems in environmental control of the animal modules, environmental control of the anechoic chambers and general air tempering in the labs/offices. Due to the failure to take occupancy in the present report period, many other facility improvements were likewise delayed. These include installation of the high peak power, pulsed klystron transmitter and the vertical feed in Chamber C. Continued problems in the basic structure and existing construction have played a large estimate at the present time is that we may occupy the building early in the first quarter of FY 80.

Another construction project to renovate present department space in the reactor room of Building 40 has progressed to the steps of architectural plans and a formal budget submission for an MCA in FY 80. The project includes division of the present reactor room into two floors. Office space will be in the lower level and laboratory space will be in the upper level. The laboratory space will permit expansion of in vitro studies and commencement of a new project in millimeter wave bioeffects.

B. Programmatic Review

This year LTC Larsen was elected chairman of the Tri-Service

Electromagnetic Radiation Panel (TERP). This involves an expanded role as TERP spokesman in the ever growing list of requests for programmatic review. Major items in that task were an Office of the Undersecretary of Defense topical review, development of a Department of Commerce task force (by direction of the Office of Science and Technology Policy) to formulate a Biological Effects of Non-ionizing Electromagnetic Radiation (BENER) national research plan, new reporting requirements for the National Telecommunications and Information Agency (NTIA), hearings for the U.S. House of Representatives Committee on Natural Resources and Environment (chaired by Congressman Ambro) and appearances before the Electromagnetic Radiation Advisory Council. These activities are, of course, in addition to program reviews within DA such as the Congressional Descriptive Summaries, Modern Army Research and Development Information System and the RDAC. Also, a DA manpower survey was taken in the present report period. To paraphrase Winston Churchill, never have so many reviewed so few!

C. Organizational Alterations

The Department of Microwave Research over the last two years has faced a mission expansion combined with a staff decrease. Although manpower analyses have verified the need for additional research staff, we are unable to recruit capable individuals. The major reason for this recruitment problem is the grades we are able to offer. The grade structure, in turn, is a function of our organizational level. These and other considerations have resulted in a two-step plan to increase our research productivity and allow us to meet further mission requirements in FY 80-82. The first step is to maximize the productivity of our present staff by instituting a plan for contractor operation of the WRAIR microwave exposure facility at Forest Glen with retention of government ownership (i.e. the so called GOCO model of facility operation). A Memorandum of Understanding was developed and the Electromagnetic Compatibility Analysis Center was designated by the Assistant Secretary of Defense as the performing agency. The plans call for a facility manager, a microwave engineer, an electronic technician and a clerk typist. This GOCO model will compensate for staff attrition and eventually relieve the burden of facility operation. We expect a transition/training period of ca. 6-12 months after which we hope to accommodate a projected increase in exposure facility utilization by both intramural and extramural users. Subsequently, those intramural staff involved with facility operation will be able to devote their time and energies to the expanding needs of the intramural program.

The second step is a proposal to reorganize at the division level pending review in FY 81. The chief advantages are staff expansion with a grade structure that allows successful recruitment and programmatic development into areas where existing staff expertise is not available. Concurrently, we plan a large expansion in the contract program to meet the urgent mission needs of FY 80.

RESEARCH ACTIVITIES

A. Dosimetry: Introduction

Dosimetric studies are among the most fundamental lines of inquiry with respect to radiofrequency radiation (RFR) bio-effects. The biological consequences of RFR exposure depend largely upon the spatial and temporal distribution of absorbed RFR energy. In this research topic, there presently exists two classes of projects: those based upon invasive methods of dosimetric analysis and those based upon non-invasive methods of dosimetric analysis. The former includes projects related to implantable, RFR transparent electrodes for biological transduction and RFR compatible telemetry. The second category contains projects related to microwave antenna developments, microwave scattering parameter measurement systems for spatially dependent insertion loss measurements, microwave differential propagation delay with high spatial and temporal resolution and scanning systems to apply these microwave devices to target characterization.

1. MIC Electrode Program: Progress

The present reporting period has been one of disappointing productivity in this area. The contractor experienced a series of strikes and walkouts which lasted over a period of approximately nine months. Plans to begin preproduction runs on the MIC temperature electrode, therefore, have been delayed to FY 80. However, the successful conclusion of the development phase of the temperature electrode may be marked by issuance of U.S. Patent #4,148,005 in the present reporting period. A publication describing this electrode did appear during the present report period.

The implantable MIC electric field probe suffered a similar fate of slow progress, but at least some preliminary development steps were accomplished. These included a demonstration of acceptable sensitivity in a uniaxial design ($20 \mu\text{V}/\text{milliwatt cm}^{-2}$) and satisfactory isolation (ca. 20 dB) between "orthogonal" arms

in a triaxis design. Thus, the antenna and bias free detector performance predictions have been verified. This is an encouraging result in that the dimensions are smaller than heretofore attempted (i.e. 25 μ). The remaining critical area of performance verification is probe sensitivity to the complex permittivity of the surrounding medium. This task will be accomplished in FY 80 when deliveries are complete.

A related project in this area is the development of an RFR compatible telemetry package. The design and development of prototype temperature encoding electronics and telemetry transmitter is complete and has been partially tested using a simulated temperature transducer. The unit has been constructed using micropower integrated circuits which provides a battery life of 200-300 hours. The physical size of the unit is 30 mm x 18 mm x 8 mm including the batteries and antenna. This unit operates in the commercial FM band (88-108 MHz) and has a range of about 5 meters.

2. Non-invasive Dosimetric Analysis: Progress

The water coupled antenna development has been the subject of U.S. Patent #4,162,500 which issued in the present report period. Also, a paper on the antenna appeared in the engineering literature. The antenna has become the element in a water coupled array for high speed data collection. The present electromechanical scanner requires ca. 270 minutes for data collection. The water coupled array will reduce the data collection time by a factor of 10 to 100. In addition to the speed afforded by electronic beam steering, the system will include focusing functions and accommodate larger targets. The array program has progressed to through the point of system configuration and performance prediction by computer aided design (CAD). The CAD results predict that -10 dB beam widths in elevation and range over beam steering angles of 60 degrees in all directions will be in the order of 6 to 10 mm within a noise floor of -125 dBm for arrays of ca. 150 elements. In all focus points, the CAD results predict worst case unwanted polarization ca. -8 to -10 dB relative to the desired polarization. Better performance occurs as scan angle is decreased to zero where unwanted polarization is ca. -40 dB. The project is now to the point of element production for the receive array while CAD studies continue for the transmitter array.

The methods of target characterization developed in the last report period have been further developed. Two papers on the subject appeared in the scientific literature during the

present report period on the scattering parameter and time delay methods as applied to phantom tissue dielectric models. The scattering parameter system has been applied to isolated canine kidney. The target was characterized by a two-dimensional spatial series of complex numbers for the magnitude and phase of scattering parameter S_{21} . The data was processed as two real-valued series to produce magnitude and phase dosimetric images. The final data demonstrated that the spatial variations of S_{21} for canine kidney do correspond to known regional specializations in function.

Microwave hardware improvements to the differential propagation delay system now allow spatial resolution limit of ca. 5 mm and a propagation delay resolution of ca. 40 picoseconds. The propagation delay system was shown to be less sensitive to multipath propagation by dielectric guiding at the organ/water bath interface. Interior details of the kidney were, of course, different for the propagation delay and scattering parameter systems, but the general comparability of results at the level of major regional specialization within the kidney was most encouraging.

From the perspective of hazards analysis, the results suggest that the outer medullary site is the one with greatest insertion loss. To the extent that this represents greater energy deposition, we may infer that high power (4 GHz) energy at the kidney will more likely effect concentration (water transport) than electrolyte transport or filtration.

3. Dosimetric Modeling: Progress

Computational studies of the spatial distribution of induced electric field in man-sized phantoms with heterogeneous dielectrics were also completed and published in this report period. These studies confirm the importance of realistic spatial variations in complex permittivity in that peak absorbed power density does vary by a factor of 60% in comparison to the homogeneous case under conditions near resonant absorption.

4. Analytical Studies of Army Radars: Progress

In the present report period, a two-year project on biohazard analysis of Army radars was concluded. The radar systems were described in terms of power density calculations for near field main beam, side lobes and back lobes of fixed aperture radiators. The systems studied were Improved Hawk, Nike, Firefinder and Roland. Main beam far field studies were

completed for Patriot. The studies were based upon recommended site configurations.

B. Dielectric Relaxation: Introduction

This programmatic line represents the most fundamental level of work within this report period. Since dielectric relaxation implies studies of the frequency dependence of complex permittivity, these topics are basic elements of any quantitative description of the microwave propagation in biological dielectrics and the mechanism of frequency dependent absorption in biosystems at the cellular and molecular level. This topic includes the development of automatic network analyzer based measurement systems for high speed permittivity analysis and the development of systems for the measurement of complex permittivity in situ. The latter is especially important to the extent that physiological responses do in fact alter the complex permittivity of tissues. Thus, it is possible that microwave exposure does itself alter the microwave properties of the exposed tissue/organ.

1. In Vitro Measurements of Dielectric Relaxation

Automatic network analyzer (ANA) based measurements were developed in the prior two report periods. In brief recapitulation, the advantages of ANA based methods are chiefly those related to speed whereby it becomes possible to collect the dispersion of the reflection coefficient at 100 frequency steps over a bandwidth from 1 to 5 octaves in a time of ca. 1 second per frequency. It is now possible to examine the temporal behavior of the dispersion during physiologic and pathologic processes with time bases in the order of one to two minutes. In prior reports, these methods were applied to the HF band (1 to 30 MHz) for examination of the β dispersion due to the cell membrane. A publication describing the HF band work appeared in this report period.

The ANA methods were extended to the microwave frequency range in the present report period. The analytical model was altered from the lumped circuit element approach in the HF band to a total loss method in the microwave range of 3-6 GHz. Performance verification has taken place for standard dielectric liquids such as water, saline solutions and various organic solvents. At the present time, studies of erythrocyte cell suspensions have begun, but the project is in too early a stage to summarize results at this time.

2. In Situ Measurements of Dielectric Relaxation

The ANA based methods were applied to a modified electrical model in order to extend its data collection advantages to the in situ setting. Prior report periods describe the electrical model which will be briefly recapitulated here: A monopole antenna radiating into a lossy dielectric medium exhibits a driving point admittance/impedance which is dependent upon the complex permittivity of the medium. Thus, a measurement of the reflection coefficient at the driving point may be used to estimate the complex permittivity of the medium in which the antenna is "imbedded". The antennas have been open-ended coaxial lines of .085" and .141" OD. Frequencies of operation vary between 1 and 4 GHz. These variously have small ground planes attached to the outer conductor and/or small probes attached to the inner conductor. The dielectric volume at the radiating element to which the method is sensitive is ca. 9 cubic mm. Prior work has established the accuracy and sensitivity of the method.

Research in the present report period centers on the effect of physiological and pathophysiological alterations on complex permittivity. The first of these explored the effects of circulatory alterations and post mortem changes in tissue permittivity. The results indicate that circulatory cessation produces a prompt decline in both dielectric constant and conductivity. This is followed by a gradual further alteration over the course of the next few hours until reasonably stable values are reached. These results have the important implication that tabled values of complex permittivity based upon extirpated necropsy specimens do not represent the microwave properties of living organs and tissues. The error involved is ca. 20-30% in both dielectric constant and conductivity.

C. Thermoacoustic Expansion: Introduction

In 1976, the Department of Microwave Research convened a group of consultants to review a recent but small body of experimental data which implicated low average power microwave radiation as an agent capable of inducing alterations in the blood-brain-barrier. The causative factor was thought to be high peak power and its consequent pressure wave. The consensus of opinion was that the experimental methods were faulted, but that the results did justify further examination of the issues. A priority order of experimentation should begin with studies of membrane permeability alterations, followed by studies of isolated capillary beds, then returning to in situ studies. On the basis

of that recommendation, two studies began in 1977-78. The first was a study of neural membrane and the second a study of erythrocyte membrane. In the former, the dependent variable was alterations in the optical properties of the neural membrane that accompany propagation of an action potential. In the latter, the dependent variables were ionic and enzymatic partition shifts from the intracellular to the extracellular space. In both cases, the experimental designs compared pulse and continuous radiation of the same average power.

Thermoacoustic expansion may be further implicated in microwave cataractogenesis. Recent ultrastructural and biochemical studies have shown that early changes in the lens fiber which involve membrane deformation and cytoskeletal alterations are common to both diabetic and thermal cataracts.

1. Neural Membrane Studies: Progress

This year's work involved developing the experimental apparatus, exposing many nerve preparations to microwave radiation, data analysis, drawing conclusions and preparation of the publication. The equipment development included reducing interference in the nerve action potential equipment, installing calcite polarizers and iris in the optical path and reducing transmission of vibration to the equipment. Also, dissection procedures for the animals were improved. Data was taken on at least 50 pairs of nerves, and was analyzed by time averaging techniques and statistical methods. The preliminary conclusions are that exposure to microwave radiation accelerates degradation of the birefringence alterations that accompany the action potential. Further, pulsed radiation appears to be more effective than continuous radiation of the same average power.

2. Erythrocyte Membrane Studies: Progress

A blood exposure system was developed that applied uniform microwave energy to a suspension of blood cells, while maintaining them at a constant temperature, with agitation but not hemolysing for a period of 30 minutes. This required electrodes of micro flatness, special sealing compounds and high stability electronic temperature regulators. The now completed pilot phase resulted in the selection of the optimum biological indicator (LDH) for the condition of the blood cell membrane. An apparatus which allows 8 samples of blood to be exposed simultaneously has just been completed, and will allow statistically significant tests for effects of microwave energy. We intend to study both continuous and pulse energy at the relaxation

frequency of the cell membrane and other non-resonant frequencies.

3. Ocular Effects: Progress

In vitro thermal studies have confirmed that these apparently precataract changes are detectable with lens temperature elevations of only 2° C as opposed to the prior assumed threshold of 4-6° C. Also the depth of heat induced membrane deformation is linearly related to the temperature elevation. One major advantage to the present methods is the use of tissue culture methods to decrease the latency between the time of injury and optical aberration in the lens fibers.

Concurrently, the microwave phase of the project began with the design and development of a transmission line system for in vitro exposures of isolated lens. The experimental procedures will compare pulse and continuous exposures of the same average power in a temperature controlled specimen chamber. The in vitro exposures are to be followed by in situ exposures after the pulsed klystron transmitter and exposure facility are operational.

D. Microwave Technology Application: Introduction

This programmatic line contains projects with no direct relationship to hazards research either at the 6.1 or 6.2 level. Rather, it is a spin-off of the microwave research program into the area of biochemistry (more specifically, neurochemistry). The goal is to provide for rapid inactivation of thermolabile enzymes in the brains of mice and rats without destruction of regional anatomy. To the extent that enzyme-substrate reactions may be examined within time frames comparable to physiological processes, it will be possible to produce biochemical correlations of numerous physiological and pathophysiological events wherein temporal sequences are a primary feature. Also, regional specialization in organs such as brain may be examined for enzyme kinetics and substrate pool variations as an aid to improved understanding of behavior and the actions of psychotropic drugs.

Two projects were active during the present report period. One of these was the conclusion of an analytical study of field induction in three-dimensional, heterogeneous dielectrics as a means to simulate design candidates for improved uniformity of heating for complex dielectrics in waveguide. The second was the beginning of a project to develop under contract an improved

inactivator. The improved inactivator is intended to replace the one in use since 1975. Its main features will be improved regulation of the net power delivered to the load, higher peak available power and improved reproducibility between exposures.

1. Analytical Studies: Progress

This project contains the first successful three-dimensional solution of the field equations for waveguides partially filled with dielectrics. The analytical approach was that of the tensor Green's function followed by thermodynamic modeling of convection/radiation cooled dielectric blocks. The thermal predicts were then validated by thermograph scans of various breakdown models of dielectric rectangles exposure in WR 284 under the presumed conditions of placement, power, etc.

The conclusions of the study were the subject of a publication and presentation during the present report period. In general, the analytical predictions correctly modeled the general patterns of field induction and heating. For example, the heating peak was at the distal end of the block and it was offset to the appropriate side. However, the detailed spatial distribution of predicted heating did not match the experimentally measured heating pattern in that the peak was offset by several millimeters (e.g., 2-5 mm). In light of the importance of distances such as 3 mm in the structure and function of brain and given the comparatively simplified dielectric, the conclusion was that analytical improvements are necessary before these methods may be successfully applied to inactivator design.

2. Hardware Development: Progress

The present report period was one of little progress in hardware development. An RFP was produced, bids were evaluated and an award was made to Georgia Institute of Technology. The first design review as held and basic system configuration was determined.

3. Cerebellar Levels of cGMP and Locomotor Activity in Rats

The effect of locomotor activity on brain regional levels of cyclic guanosine 3', 5' - monophosphate (cGMP) and cyclic adenosine 3', 5' - monophosphates (cAMP) was studied in collaboration with investigators from the Department of Neurosciences utilizing the technique of rapid microwave inactivation of brain enzymes. Following 5 minutes of running in an activity

wheel, there was a two-fold elevation over control levels of cerebellar cGMP. Significant elevations were seen in eight other brain regions. No changes were observed in cAMP. Locomotor activity may contribute to elevations in cGMP seen in brains of rats exposed to a variety of stressful conditions.

E. Behavioral Program: Introduction

This program line has been denied access to the anechoic chambers at our exposure facility in Forest Glen as a result of the continued delay in completion of the renovation. Transmission line exposure systems have been acquired to fill that need. These projects will utilize a circular waveguide exposure apparatus. Insofar as it is a transmission line exposure system, unrestrained animal subjects are exposed to circularly polarized microwave fields that minimize field perturbations due to subject movement and allow for continuous accurate measurement of energy absorption by the power difference method.

1. Behavioral Project Plans:

a. Behavioral Detection and Discrimination

This project will investigate microwave/central nervous system interactions by determining the limits of behavioral detection and discrimination of simple and complex microwave signals by laboratory animals. Operant behavioral techniques will be utilized for the psychophysical assessment of detection thresholds and discrimination functions for continuous and pulsed waveform signals.

b. Blood-Brain-Barrier Psychopharmacology

Operant psychopharmacological techniques will be utilized to develop behavioral baselines in laboratory animals which should be sensitive in indicators of drug/microwave interactions. Possible mechanisms include drug/receptor alterations and/or alterations in the permeability of the blood-brain-barrier to specific drug agents. Studies will employ standard operant paradigms and will focus on behavioral changes induced by continuous or pulsed waveform microwave signals.

c. Conditioned Taste Aversion

Previous work in this laboratory has utilized the conditioned taste aversion technique for determining the levels at which microwave radiation acquires aversive motivational

properties. Microwave thermalization has been compared with heat stress as the unconditioned stimulus (see FY 78 Annual Report). Additional work has been conducted during FY 79 which expands the usefulness of this technique as a research tool for assessment of microwave effects on central nervous system and behavior.

Rats with surgical interruptions of the mandibular branch of the trigeminal nerve were studied for their ability to acquire and retain conditioned taste aversion. These studies showed that denervated rats acquire and extinguish taste aversion in a manner comparable to controls, but show lower intake of a palatable solution of saccharin-flavored water. Partially trigeminal denervated animals exhibit normal detection threshold and aversion curves, however, lesioned animals drank significantly less than controls at the highly preferred concentrations. Elimination of sensory information except taste from the lower portion of the mouth appears to reduce the incentive value of a normally preferred substance.

d. Comparison of Free Field and Transmission Line Exposures

Plans were developed for a comparison of transmission line (circular waveguide) and free field (anechoic chamber) exposure conditions. Since the wavelength of the incident radiation in the transmission line is ca. 3 times longer than that in free space at the same frequency, there are significant questions about the comparability of experimental results under the two circumstances and the generality of findings in transmission line exposures.

e. Energy Absorption and Distribution

With completion of the Bldg 502/503 research facility, research on measurement technologies for energy absorption and distribution in experimental animals and models simulating humans will be resumed. These include further development of whole-body absolute calorimetry for determining average whole-body dose and the in vivo testing of the radio transparent electrodes for temperature and tissue electric fields induced by RF irradiation.

f. Behavioral and Nervous System Effects

With completion of the Bldg 502/503 research facility, research on effects of plane wave fields in various

field configurations to investigate low level radiation effects on behavior and nervous system function will be resumed. Such studies will include further research on changes in excitability with high peak power pulsed fields, phenomenology of field escape behavior and effects of stressors (such as high ambient humidity) in combination with RF exposure.

F. Electronic Support: Introduction

This section reports major products of the electronic support staff. These items were applied to various research topics as indicated, but the instrument development was itself sufficiently difficult and/or innovative that special attention is merited. The products represent three classes of instruments: display devices; data recording; and data acquisition.

1. Display Devices

During the report period, the principle project completed was the 3-D display system. It consists, physically, of two units: one oscilloscope-type vector display and one control console. The latter contains all the electronics controls to simulate 3-D images, test patterns and interface to three 16 bit parallel outputs from a computer. This is a highly complex system that uses analog techniques to magnify images, perform zoom functions and rotate perspective. This in-house development resulted in a considerable cost saving over commercially available units. The application intended is for use with the non-invasive dosimetric projects.

2. Data Recording

A second unit completed during this period was a scope-camera trigger-unit. It permits remote triggering of a type C-5-A camera from a microwave-power meter. This to facilitate single events, such as the resulting scope trace of a single, short microwave exposure. The intended application was for variable latency (relative to power application) thermography in support of the MIC electrode program.

3. Data Acquisition

A third device completed was a microwave sweep oscillator. By purchasing a relatively inexpensive YIG oscillator unit and adding a linear sweep circuit to it, developed at the electronics shop, we were able to construct a highly linear, very fast microwave sweep oscillator that operates in the 2 GHz-4 GHz

range. The commercial oscillators are about 15 times slower, and have serious problems with hysteresis and non-linearity. The intended application is to serve as the microwave drive source for the differential propagation delay measurement system. The improved speed and linearity will directly contribute to improved time delay resolution.

Project 3E162771A805 MICROWAVE INJURY

Work Unit 041 Biological Interactions with and Hazards of Microwave
Radiation

Literature Cited.

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Project 3M362750A808
DRUG AND VACCINE DEVELOPMENT

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a		2 DATE OF SUMMARY ^a		REPORT CONTROL SYMBOL DD FORM (AR) 1498	
3 DATE PREV SUMMARY		4 KIND OF SUMMARY		5 SUMMARY SCTY ^a		6 WORK SECURITY ^a		7 REGRADING ^a	
78 10 01		D. Change		U		U		NA	
8 AGENCY ACCESSION ^a		9 AGENCY ACCESSION ^a		10 AGENCY ACCESSION ^a		11 AGENCY ACCESSION ^a		12 AGENCY ACCESSION ^a	
78 10 01		D. Change		U		U		NA	
13 NO / CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
A. PRIMARY		63750A		SM362750A808		00		001	
B. CONTRIBUTING		CARDS 114F							
11. TITLE (Precede with Security Classification Code) ^a									
(U) Phase II Antimalarial Drug Trials									
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ^a									
Clinical Pharmacology									
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD			
78 10		CONT		DA		C. In-house			
17 CONTRACT/GRANT		18 DATES/EFFECTIVE:		EXPIRATION:		19 RESOURCES ESTIMATE		20 PROFESSIONAL MAN YRS	
A. NUMBER ^a		NA				FISCAL YEAR		3	
B. TYPE:		C. AMOUNT:		F. CUM. AMT.		79		180	
D. KIND OF AWARD:						80		75	
19. RESPONSIBLE DOD ORGANIZATION					20. PERFORMING ORGANIZATION				
NAME ^a Walter Reed Army Institute of Research					NAME ^a Walter Reed Army Institute of Research				
ADDRESS ^a Washington, DC 20012					ADDRESS ^a Washington, DC 20012				
RESPONSIBLE INDIVIDUAL					PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)				
NAME ^a RUSSELL, COL P.					NAME ^a HEIFFER, Dr. M. H.				
TELEPHONE: 202-576-3551					TELEPHONE: 202-576-3014				
21. GENERAL USE					SOCIAL SECURITY ACCOUNT NUMBER:				
Foreign intelligence not considered					ASSOCIATE INVESTIGATORS				
					NAME: DOBERSTYN, LTC B.				
					NAME: PAMPLIN, MAJ C.				
22. KEYWORDS (Precede each with Security Classification Code) (U) Clinical Pharmacology; (U) Phase II Efficacy; (U) Antimalarial Drugs; (U) Human Volunteer									
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number, precede text of each with Security Classification Code)									
23. (U) The technical objective of this work unit is to evaluate the effectiveness of new antimalarial drugs in non-immune human volunteers experimentally infected with malaria. Studies are performed in support of the Army antimalarial drug development program, and as an essential part of each official Investigational New Drug submission.									
24. (U) Normal male volunteers are recruited from the civilian population of the greater metropolitan Washington, D.C., area by public advertisement. Each individual is then evaluated medically. A valid informed consent to participate in the study is obtained. As a study subject, the individual is admitted to an in-patient research unit, inoculated with malaria and treated with the drug or drugs specified in the protocol for each study. Each subject is then observed for a sufficient period of time to ensure that he is cured of malaria and free from any adverse effect from his participation in the study.									
25. (U) 78 10 - 79 09 WR 171,669 was tested for efficacy against blood-induced Plasmodium falciparum Smith Strain in eight volunteers. All were cured. The same drug cured both cases of blood-induced Plasmodium vivax Chesson Strain treated. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.									

^aAvailable to contractors upon originator's approval

1954

DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498 B, 1 MAR 69 (FOR ARMY USE) ARE OBSOLETE.

Project 3M362750A808 DRUG AND VACCINE DEVELOPMENT

Work Unit 001 Phase II Antimalarial Drug Trials

Investigators:

Principal: Melvin H. Heiffer, Ph.D.

Associate: MAJ R. Desjardins, MAJ C. Pamplin, MAJ T. Cosgriff, COL C. Canfield, LTC B. Doberstyn, MAJ B. Schuster

1. Description.

Phase II studies in human volunteers are concerned with evaluating efficacy of drugs in a limited number of patients. This type of study is the essential bridge between the determination of drug tolerance in humans and the wide scale study of the new drug in human patients. Phase II studies on new antimalarial compounds involve determining the drug effect on the course of induced malaria infections in volunteers.

2. Evaluation of WR 171,669 in the treatment of multi-drug resistant Plasmodium falciparum malaria.

a. Background:

Antimalarial chemotherapy has historically occupied a position of great interest and concern to the medical community and the world at large. Quinine was the sole effective treatment for malaria until intensive research in the United States during the Second World War resulted in the discovery of chloroquine, a much more effective drug. The introduction of chloroquine raised hope for worldwide eradication of the disease. However, not only have eradication efforts proved futile, but today 2/3's of the world's population remain at risk. In Southeast Asia and parts of South America the majority of P. falciparum parasites have developed stable resistance to chloroquine and most other available antimalarials. This resistance is of enormous significance.

P. falciparum, Vietnam Smith, is resistant to chloroquine, inhibitors of dihydrofolate reductase and is relatively resistant to quinine, sulfonamides, and sulfones. The blood sample used in this study was obtained from Case No. 506, H.S. Truman Laboratory, Kansas City, Missouri, on April 3, 1975. The sample is HB antigen and antibody negative and has 16,500 parasites/mm³.

Encouraging results prompted the selection for clinical trials of a phenanthrenemethanol, WR 171,669. In Phase I studies, gastrointestinal symptoms of nausea, cramps and diarrhea limited single day oral dosage of WR 171,669 to 1250 mg. However, no physical or laboratory abnormalities, phototoxicity, or gastrointestinal blood loss were noted in subjects receiving this drug (Rinehart et al., 1976). Subjects receiving WR 171,669, 250 mg four times per day, became symptomatic after 4-5 days of dosing. In initial Phase II studies, WR 171,669 given orally at 1 gram/day for 3 days cured 6/6 subjects with chloroquine-resistant (Vietnam Smith) and 3/3 cases of chloroquine-sensitive (Uganda I) infections (Rinehart et al., 1976).

These preliminary Phase II studies have shown WR 171,669 to be an effective antimalarial agent. However, before field trials can be undertaken, further Phase II studies are needed to confirm cure rates and optimize dosage schedules in non-immune individuals.

b. Methods:

i. Selection of subjects:

Subjects for this study were recruited from the Military Research Volunteer Subject (MRVS) population at Ft. Detrick and from the non-military population of the Washington, D.C. area. These subject populations primarily include people from religious groups, college students, medical students and graduate students. The non-military subjects were recruited by public advertisement (newspapers, bulletin boards) and paid by JAG approved government contractual procedures. The military subjects were recruited from the MRVS population at Ft. Detrick by non-coercive local announcements according to existing Army Regulations.

Healthy male subjects, ages 21-50, from 50-100 kg in weight, were selected for this study. Prospective subjects were given a thorough medical history review, physical examination and laboratory screening at Walter Reed, including chest x-ray, visual acuity (Snellen chart), audiometry, electrocardiogram, CBC and platelet count, BUN, creatinine, total protein and A/G ratio, SGPT, SGOT, alkaline phosphatase, Na, K, Cl, CO₂, FBS, urinalysis and HB_sAg, HB_sAb, by RIA. Minimum criteria for rejection included, but were not limited to, a history of malaria, hepatitis, splenectomy, significant lymphadenopathy or organic heart murmur or any significant laboratory abnormality.

ii. Informed consent:

Clinically acceptable subjects were fully informed of the details of the procedures and inherent risks according to guidelines set forth in AR 70-25 and AR 40-7. Following discussion with the investigators, pursuant to the above, and reading of the Subject Information Sheet, written consent was obtained.

iii. Experimental design:

Three treatment groups were studied. A minimal curative dose regimen was sought for WR 171,669. Subjects were inoculated with the Vietnam Smith Strain and treated at each of the following dose levels in sequence:

<u>WR 171,669 RX Levels</u>	<u>Number of Subjects</u>
250 mg q6hrs x 3 days	3
250 mg q6hrs x 2 days	3
250 mg q6hrs x 1 day	2

A dosage regimen less than 100% effective is no longer acceptable in view of the recent success of the Army Antimalarial Program with another class of drug (mefloquine). Therefore, when a single failure occurs, i.e., a subject is not cured, the testing sequence is discontinued, the patient treated with 1.5 gm mefloquine administered as a single dose and subsequently followed as outlined elsewhere.

iv. Clinical procedures:

Screening procedures were performed at one to three weeks before transfer to Ward 200, USAMRIID. Vital signs and temperature were recorded at least four times per day during hospitalization.

After hospitalization the subject was infected with P. falciparum. Each subject was inoculated with a thawed deglycerolized specimen of the Vietnam Smith strain. These specimens have tested negative for Australian Antigen.

After inoculation, the subjects remained on Ward 200, USAMRIID, Fort Detrick, Maryland, and were examined daily. Five days after inoculation, daily specimens of blood were obtained for parasite counts. When patency developed or clinical

symptoms appeared parasite counts were done twice daily and the battery of laboratory tests were done every three days. When parasitemia was confirmed by two positive thick smears, oral treatment with WR 171,669 was initiated.

After peripheral blood smears were negative for malaria parasites for at least 5 days and the individual was asymptomatic, he was discharged from the hospital for outpatient follow-up at WRAIR. Physical examination and thick smears were then done weekly thereafter for eight weeks. Clinical chemistry, CBC and urinalysis were repeated at Walter Reed every other week for eight weeks. RIA for HB_sAg and HB_sAb were repeated every two weeks for three months and monthly for three additional months.

Drug efficacy was evaluated by calculating cure rate, parasite and fever clearance times.

c. Results:

The results obtained thus far with treatment of malaria induced with the Smith strain of P. falciparum are presented in Table 1. To date, WR 171,669 has resulted in cures in at least five of the eight cases. Cure is defined as elimination of parasites from the blood, disappearance of signs and symptoms of malaria, and lack of recrudescence during the 60 day follow-up period. Three volunteers have been followed for less than 60 days, but are still parasite-free.

3. Evaluation of WR 171,669 in the treatment of Plasmodium vivax malaria.

a. Background:

In order to predict the activity of new antimalarial drugs against naturally acquired infections, the drugs were tested first against several strains of P. falciparum and P. vivax which had been characterized with respect to drug resistance and the clinical course of illness they produced in man. Testing against these specific strains has become essential for the clinical evaluation of new antimalarial drugs, permitting indirect comparisons with many standard and investigational drugs tested in the past. Thus, it was proposed to test the blood schizonticidal activity of WR 171,669 in P. vivax (Chesson strain) malaria.

The Chesson strain of P. vivax was first isolated during WW II from a SGT Chesson who had returned from duty in

New Guinea. It is characterized by a series of relapses occurring at short intervals following a primary mosquito-induced infection. This is in contrast with the characteristically unreliable incubation periods and long relapse intervals of other characterized strains. Because of this property of a reliably short incubation period and early relapse time of 40-60 days, this strain has remained for more than 30 years the most important strain of its species for the study and development of antimalarial drugs in man. It is a strain which closely mimics the P. vivax found in Southeast Asia.

The specimen of Chesson strain P. vivax used in these studies was rejuvenated from a specimen obtained from a volunteer at the Maryland House of Corrections. Blood from this volunteer was cryopreserved in October 1974. On 26 May 1976, the frozen specimen of blood was transported from WRAIR to USAMRIID, where it was immediately thawed and deglycerolized and a volunteer subject was inoculated with 5 cc of re-suspended cells, containing approximately 5×10^6 parasites. On 11 June 1976, the volunteer developed clinical malaria. The next day, 200 ml of blood was drawn from the patient, who had a parasite count of approximately 200/mm³, and transported to WRAIR on wet ice. At WRAIR the blood was immediately glycerolized and quick frozen in liquid nitrogen. The subject was cured with a single oral dose of mefloquine (1.5 gm). The parasite clearance time was 66 hours and the fever clearance time was 61 hours. His specimen, now preserved in liquid nitrogen, should remain viable indefinitely and is the source of the inoculum for this study. A six-month follow-up of this donor subject revealed no clinical or serological evidence of hepatitis.

Encouraging results prompted the selection for clinical trials of another phenanthrenemethanol, WR 171,669. In Phase I studies, gastrointestinal symptoms of nausea, cramps and diarrhea limited single day oral dosage of WR 171,669 to 1,250 mg. However, no physical or laboratory abnormalities or phototoxicity were noted in subjects receiving this drug. Subjects receiving WR 171,669, 250 mg four times per day, developed these same gastrointestinal symptoms after 4-5 days of dosing. In initial Phase II studies, WR 171,669 was given orally in doses of 1 gm/day for three days and cured six out of six subjects with chloroquine-resistant (Vietnam Smith) and three out of three cases of chloroquine-sensitive (Uganda I) infections (Rinehart et al., 1976).

These preliminary Phase II studies have shown WR 171,669 to be an effective antimalarial agent. However, before field

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trials can be undertaken, further Phase II studies are needed to confirm cure rates and to optimize dosage schedules in non-immune individuals. In addition, it is necessary to test the effectiveness of WR 171,669 against vivax malaria because of problems associated with current therapy.

b. Methods:

i. Selection of subjects:

Subjects for this study were recruited from the Military Research Volunteer Subject (MRVS) population at Ft. Detrick and from the non-military population of the Washington, D.C., area. These subject populations primarily include people from religious groups, college students, medical students and graduate students. The non-military subjects were recruited by public advertisement (newspapers, school bulletin boards, etc.) and paid by JAG approved government contractual procedures. The military subjects were recruited from the MRVS population at Ft. Detrick by non-coersive local announcements according to existing Army regulations. Healthy white male subjects, ages 18-50, from 50-100 kg in weight, were selected for this study. (The study is limited to caucasians because of the known decreased susceptibility of blacks to P. vivax infection.) Prospective subjects were given a thorough medical history review, physical examination and laboratory screening at USAMRIID or Walter Reed, including chest x-ray, visual acuity (Snellen chart), audiometry, electrocardiogram, CBC and platelet count, BUN, creatinine, total protein and A/G ratio, SGPT, SGOT, alkaline phosphatase, Na, K, Cl, CO₂, FBS, G-6-PD assay, urinalysis and HBsAb, HBsAg by RIA. Minimum criteria for rejection included, but were not limited to, a history of malaria, hepatitis, splenectomy, significant lymphadenopathy or organic heart murmur, or any significant laboratory abnormality, including decreased G-6-PD activity.

ii. Informed consent:

Clinically acceptable subjects were fully informed of the details of the procedures and inherent risks according to the guidelines set forth in AR 70-25 and AR 40-7. Following discussion with the investigators pursuant to the above and reading of the subject information sheet, written consent was obtained.

iii. Experimental design:

One treatment group was studied. Two subjects inoculated with the Chesson Strain who became patent were treated with WR 171,669, 250 mg every 6 hours for 3 days.

A dosage regimen found to be less than 100% effective is no longer acceptable in view of the recent success of the Army Antimalarial Program with another class of drug (mefloquine). Therefore, when a single failure occurs, that is, a subject is not cured, the testing sequence is discontinued and the patient treated with a standard regimen of 1.5 gm of chloroquine administered over 3 days (the treatment of blood induced P. vivax with primaquine is not required, since exoerythrocytic forms do not develop).

iv. Clinical procedures:

Screening procedures were performed one to three weeks before transfer to Ward 200, USAMRIID. Vital signs and temperature were recorded at least four times per day during hospitalization.

After hospitalization, the subject was infected with P. vivax (Chesson strain). Each subject was inoculated with a thawed deglycerolized specimen of the strain. The deglycerolization procedure performed at USAMRIID consists of a stepwise washing of the specimen using a sterile technique with USP sterile saline and hypertonic glycerol with four decreasing concentrations of glycerol until the final specimen for inoculation is suspended in 10 cc of isotonic USP sterile saline. This stepwise procedure is necessary to maintain viability of the parasites. A 0.1 cc aliquot of the final inoculum is frozen at -20°C for 60 days for potential retrospective microbiological studies in the event of unexplained febrile illness.

The subjects were quartered on Ward 200, Ft. Detrick, Maryland, and examined daily. Five days after inoculation, daily specimens of blood were obtained for parasite counts. When patency developed or clinical symptoms appeared, parasite counts were done twice daily and the laboratory tests outlined elsewhere done every three days.

When parasitemia was confirmed by two positive thick smears, oral treatment with WR 171,669 was initiated.

After peripheral blood smears were negative for malaria parasites for at least five days and the individual was asymptomatic, he was discharged from the hospital for outpatient followup at USAMRIID or WRAIR. Physical examination and thick smears were done weekly thereafter for eight weeks. Clinical chemistry, CBC and urinalysis were repeated at Walter Reed every other week for eight weeks.

When the subject failed to become parasitemic within three weeks after inoculation, presumptive cure with 1.5 gm chloroquine was given and he was followed for 8 weeks.

Drug efficacy was expressed as cure rate, parasite and fever clearance times.

c. Results:

Six volunteers were inoculated with the Chesson strain of Plasmodium vivax. Only two of the volunteers became patent. The others were treated with a standard course of chloroquine phosphate when patency failed to develop. The results obtained thus far with treatment of malaria induced with the Chesson strain of P. vivax are presented in Table 2. Both cases are considered cures.

Table 1
Phase II Studies on the Effects of NR 171,669 for the Treatment of
Blood-Induced Plasmodium falciparum Smith Strain in Volunteers

Patient Number	Dose	Toxicity	Parasite Count at Treatment	Highest Parasite Count	Parasite Clearance Time	Fever Clearance Time	Followup Period
1	250 mg Q6H x 12	None	220/mm ³	222/mm ³	45 hr	102 hr	60 days
2	"	"	1200/mm ³	1200/mm ³	70 hr	119 hr	"
3	"	"	1620/mm ³	1620/mm ³	82 hr	91 hr	"
4	250 mg Q6H x 8	Mild diarrhea	230/mm ³	230/mm ³	28 hr	50 hr	"
5	"	None	190/mm ³	600/mm ³	40 hr	110 hr	"
6	"	"	150/mm ³	430/mm ³	63 hr	116 hr	47 days
7	250 mg Q6H x 4	"	270/mm ³	280/mm ³	69 hr	140 hr	"
8	"	"	230/mm ³	420/mm ³	93 hr	150 hr	"

Table 2

Phase II Studies on the Effects of WR 171,669 for the Treatment of Blood-Induced
Plasmodium vivax Chesson Strain in Volunteers

Patient Number	Dose	Toxicity	Parasite Count at Treatment	Highest Parasite Count	Parasite Clearance Time	Fever Clearance Time	Followup Period
1	250 mg Q6H x 12	None	850/mm ³	1040/mm ³	64 hr	86 hr	60 days
2	250 mg Q6H x 12	Mild nausea, diarrhea	700/mm ³	1010/mm ³	69 hr	74 hr	60 days

Project 3M362750A808 DRUG AND VACCINE DEVELOPMENT

Work Unit 001 Phase II Antimalarial Drug Trials

Literature Cited.

References:

1. Rinehart, J., Arnold, J., and Canfield, C.J.: Evaluation of two phenanthrenemethanols for antimalarial activity in man: WR 122,455 and WR 171,669. Amer. J. Trop. Med. Hyg. 25:769-774, 1976.

Publications:

1. Doberstyn, E.B., Teerakiartkamjorn, C., Andre, R.G., Phintuyothin, P., and Noeypatimanondh, S.: Treatment of vivax malaria with sulfadoxine-pyrimethamine and with pyrimethamine alone. Trans. Royal Soc. Trop. Med. Hyg. 73:15-17, 1979.

2. Doberstyn, E.B., Phintuyothin, P., Noeypatimanondh, S., and Teerakiartkamjorn, C.: Single-dose therapy of falciparum malaria with mefloquine or Fansidar (pyrimethamine-sulfadoxine). Bull. World Health Org. In press.

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Project 3E162780A843
MEDICAL SYSTEMS IN CHEMICAL DEFENSE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^a	3 REPORT CONTROL SYMBOL (DD FORM 1498-1)	
4 DATE PREV SUMMARY	5 KIND OF SUMMARY	6 SUMMARY SCTY ^a	7 WORK SECURITY ^a	8 AGENCY ACCESSION	9 DATE OF SUMMARY	10 REPORT CONTROL SYMBOL	
78 10 01	D. Change	U	U	DA OC 6478	79 10 01		
				11 REGRADING ^a	12 DISSEM INSTR ^a	13 SPECIAL DATA CONTRACTOR ACCESS (YES) (NO)	14 LEVEL OF SUM A. PREPARED
				NA	NL		
15 NO / CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62780A	3E162780A843	00	001			
b. CONTRIBUTING							
c. CONTRIBUTING	Cards 114F						
11. TITLE (Precede with Security Classification Code) ^a							
(U) The Synthesis of Antiradiation Drugs							
12. SCIENTIFIC AND TECHNOLOGICAL AREA ^a							
012100 Organic Chemistry							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
78 10		CONT.		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PREVIOUS		b. FUNDS (in thousands)	
c. NUMBER ^a				79		1	
d. TYPE.				FISCAL YEAR		75	
e. KIND OF AWARD:				80		1	
f. CUM. AMT.						74	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Precede with DD FORM 1498-1)			
NAME: RUSSELL, P., COL				NAME: Sweeney, T.R., Ph.D.			
TELEPHONE: (202) 576-3551				TELEPHONE: 202/576-3731			
22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Klayman, D.L., Ph.D.			
				NAME:			
23. KEYWORDS (Precede EACH with Security Classification Code) ^a (U) Antiradiation Drugs; (U) Drug Development; (U) Aminoalkylthiols; Aminoalkylphosphorothioates							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Precede individual paragraphs identified by number. Precede text of each with Security Classification Code) ^a							
23. (U) The objective is to manage, integrate, and provide technical direction for both a contract and in-house program to obtain potentially active antiradiation compounds for military use through rational organic syntheses.							
24. (U) Necessary research areas are defined, proposed research evaluated, ongoing research guided, evaluated, and integrated with the other program elements. Technical advice is obtained through an Ad Hoc Study Group on Medicinal Chemistry. Information is exchanged by contractors through technical meetings.							
25. (U) Synthesis of potential antiradiation agents has been confined to latentiated WR 2721 and the no-nitrogen type agents. No testing data have been obtained. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.							

Project 3E162780A843 MEDICAL SYSTEMS IN CHEMICAL DEFENSE

Work Unit 001 Synthesis of Antiradiation Drugs

Investigators:

Principal: Thomas R. Sweeney, Ph.D.

Associate: Daniel L. Klayman, Ph.D.

The main thrust of this recently initiated program is to design and synthesize effective antiradiation drugs that will be effective by oral administration and at least maintain the dose reduction factor obtained with WR 2721.

Two research areas have been pursued during the year. One is the latentiation of WR 2721 in such a way that the active moiety will be released in vivo after oral administration. The second is the investigation of the so-called "no nitrogen" class of agents. The latentiation effort has involved difficult problems in product purification. The testing of the "no-nitrogen" target compounds has not yet been carried out.

In addition to the above areas, work on the synthesis of certain amidinium disulfides and trithiocarbonate blocking of essential thiol has just been initiated.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a		2. DATE OF SUMMARY ^a		REPORT CONTROL SYMBOL DD FORM 1498, 1 MAR 68	
3. DATE PREVIOUS ^a		4. KIND OF SUMMARY		5. SUMMARY SCTY ^a		6. WORK SECURITY ^a		7. REGRADING ^a	
78 10 01		D. Change		U		U		NA	
8. DIS'N INSTR ^a		9. SPECIFIC DATA ^a		10. CONTRACTOR ACCESS ^a		11. LEVEL OF SUB ^a		12. A 1-1-1-1-1-1	
NL		YES		NO		A 1-1-1-1-1-1			
13. NO / CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
A. PRIMARY		62780A		3E162780A843		00		002	
B. CONTRIBUTING									
C. CONTRIBUTING		CARDS 114F							
11. TITLE (Precede with Security Classification Code) ^a									
(U) CHEMOPROPHYLAXIS OF IONIZING RADIATION INJURY									
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a									
002600 Biology; 012600 Pharmacology									
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD			
78 10		CONT		DA		C. In-House			
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS		20. FUNDS (in thousands)	
A. DATES/EFFECTIVE: NA				B. PREVIOUS		C. CURRENT		D. FUTURE	
A. NUMBER ^a				FISCAL YEAR		79		0.3	
C. TYPE				CURRENCY		80		0.3	
A. KIND OF AWARD:				F. CUM. AMT.		16			
19. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION					
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research					
ADDRESS: Washington, DC 20012				ADDRESS: Washington, DC 20012					
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. Academic Institution)					
NAME: RUSSELL, Philip K., COL				NAME: DAVIDSON, David E., COL					
TELEPHONE: (202) 576-3551				TELEPHONE: (202) 576-2292					
22. GENERAL USE				ASSOCIATE INVESTIGATORS					
Foreign intelligence not considered				NAME: Grenan, Marie M.					
23. KEYWORDS (Precede EACH with Security Classification Code) ^a (U) Ionizing Radiation; (U) Chemoprophylaxis; (U) Drug Development; (U) Chemistry; (U) Toxicology; (U) Radiation Protection									
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Furnish individual paragraphs identified by number. Precede last of each with Security Classification Code) ^a									
23. (U) To find new drugs with chemoprophylactic activity against ionizing radiation injury, which can be used by military personnel in the event of exposure.									
24. (U) Selected chemical compounds synthesized by the Army program will be tested for radioprotective activity in gamma-irradiated mice. Differences in radioprotective activity, toxicity and pharmacology effected by various chemical analogs, products and formulations will be evaluated in mice.									
25. (U) A research plan and detailed protocols have been completed. Radiation equipment for rodent exposures has been acquired and installed, and safety checks and dosimetry are being performed. Nine compounds have been selected for study and are being synthesized in kilogram quantity. A study to determine the ability of WR 2721 to protect mice against neutron irradiation is in progress. No additional animal studies have been initiated to date. A computer analysis of the utility of real and hypothetical radioprotective drugs in 3 tactical nuclear scenarios has been completed. Significant reduction in the number of radiation casualties was observed, and significant number of personnel were protected to an extent that prolonged combat effectiveness could be anticipated. For Technical Report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.									

^aAvailable to contractors upon originator's approval

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Project 3E162780A843 MEDICAL SYSTEMS IN CHEMICAL DEFENSE

Work Unit 002 Chemoprophylaxis of Ionizing Radiation Injury

Investigators:

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1. Description:

Efforts to develop radioprotective drugs or combinations of drugs for use in protecting military personnel against radiations from nuclear weapons' attacks are being resumed. WR 2721, the best candidate protective drug developed by the WRAIR Program prior to 1970, had greatly improved radioprotective activity by intraperitoneal administration, providing a dose reduction factor of 2.7 in laboratory mice, a three-hour duration of protection, and improved tolerance compared to earlier radioprotective amino-thiols. The protective activity of WR 2721 after oral administration was poor in mice and non-existent in subhuman primates. This shortcoming must be overcome if this protective compound is to be considered as a candidate for use by military populations by self-administration.

Among the objectives of the new program is the development of orally effective radioprotectors. Approaches will include pro-drug synthesis and formulation efforts with WR 2721 and its close analogs, and investigation of chemical analogs of known superior experimental compounds from the former program. The new program will also investigate the ability of the newer drugs to protect against neutron irradiation.

2. Progress:

a. Radiation Protection Studies in Mice

Extensive preparations have been made for this project, but actual animal experimentation has not been initiated. A comprehensive research plan has been developed, and initial protocols have been written.

A cesium-137 small animal irradiator has been installed which will be used for rodent irradiations, and dosimetry and safety testing are in progress.

Nine radioprotective compounds have been selected from among those developed in the former program, and these are being synthesized in kilogram quantity:

WR 638	WR 77913
WR 2529	WR 151,327
WR 2721	WR 168,643
WR 3689	WR 176,542
WR 44923	

These particular compounds have been selected for further animal studies because of their potent radioprotective activity and superior tolerance. WR 3689, WR 44923, WR 151327, WR 168643 and WR 176542 have oral protective activity in mice superior to WR 2721.

b. Computer Analysis of Radioprotective Drug Utility in Three Tactical Scenarios

(1) Scenarios. The scenarios used to evaluate the utility of radioprotective drugs are those employed in the USACAA study INCA 77 (1) and the TRASANA SCORES nuclear extension (2). Weapons laydown IA and ID were used for the TRASANA study. This evaluation, therefore, is based on two force laydowns challenged with three weapons.

(2) Model. The computer model NUCAS II was used to determine the number of casualties from each scenario. This model computes the number of casualties suffering blast, thermal and initial radiation injury as well as the number suffering from combinations of two or more of these.

(3) Analysis. The dose reduction factors (DRF) selected for the analysis represent DRF's obtained experimentally in mice for either real or hypothetical radioprotective compounds. In mice, cysteine, an early radioprotective drug, had a DRF of 1.5 against gamma radiation and 1.1 against neutrons. WR 2721 had a DRF of 2.7 in mice against gamma radiation, and is represented in the analysis by a DRF of 3.0. Its protective activity against neutrons has been demonstrated, but the DRF is unknown. Although DRF of 5.0 has never been attained in vivo with any experimental drug, this value was included to demonstrate what the benefits would be if a protector with a higher DRF could be developed. Two simplifying assumptions persist throughout the analysis: that the drugs used have no effect other than changing the biological response to ionizing radiation and the neutron/gamma relative biological effectiveness (RBE) is unity. These

assumptions have not been verified experimentally in man. It is also assumed that the protective drug was taken before the attack, and that all personnel were fully protected throughout the attack.

(4) Results

The usefulness of radioprotective drugs can be expressed in two ways - as an increase in survival following exposure to nuclear radiation or as an increase in unit effectiveness for some critical period following exposure. Commanders are interested in both viewpoints and both were examined.

Table 1 shows, as a function of scenario and dose reduction factor (DRF), the number of casualties in several initial radiation exposure bands. These casualties all have sufficiently severe injuries to require hospital admission and treatment. The injuries may be due to initial nuclear radiation, blast effects, thermal effects, or combinations of two or more of these effects. Those shown to have an initial nuclear radiation dose less than 600 rads have a high probability of survival (but not early return to duty) when given proper medical care. Those receiving more than 600 but less than 1000 rads have little chance of survival, even with intensive medical care, but can be expected to survive for a period of 2-3 weeks, and to be capable of some level of performance throughout all but the terminal portion of that period. At doses of 1000-3000 rads, survival of no more than 3-5 days can be expected regardless of the level of medical care provided and performance beyond 2-3 days is improbable. Above 3000 rads, immediate neurological incapacitation is expected, and such casualties have to be considered immediately non-functional.

The reduction in the number of casualties effected by radioprotective drugs of various DRF's can be observed in the "total casualties" column of Table 1. With increasing DRF, the number of casualties is reduced. This reduction in total casualties represents the increase in the number of individuals receiving less than a 150 rad dose provided that the individuals had no burn or blast injury. Thus the reduction in total casualties shown in Table is an underestimation of the total benefits of the radioprotective drugs because the life-saving protective effect for individuals who also have casualty-producing blast or burn injuries is not taken into account.

Table 1 indicates significant increases in the number of casualties in all of the radiation bands below 3000 rads. This effect of the radioprotective drugs is due to reduction of the

number of casualties immediately incapacitated by doses of radiation in excess of 3000 rads. The significance of this beneficial effect of radioprotective drugs is that it enables many casualties to perform for periods of time ranging from hours to weeks instead of being promptly incapacitated. Thus these casualties, although many will ultimately die, may be capable of contributing to the short-term post-attack operations of their units.

The gains in numbers of personnel in various radiation bands produced by radioprotective drugs in each of the three scenarios are presented in Table 2. In this table only radiation casualties are considered. Casualties receiving burn or blast injuries in addition to radiation injuries are not considered in this table, although such casualties would also benefit if they were protected. The individuals in Table 2 are thus true gains in effective personnel, because their radiation injury is not complicated by other injuries.

The category "no effect" refers to those personnel having a radiation exposure of less than 150 rads and no other injury. The "non lethal" category includes (rather arbitrarily) those with a radiation dose less than 600 rads and having no other significant injury. The "effective for hours" category refers to those personnel having a radiation dose less than 1000 rads with no other injury and the "effective for minutes" category refers to those receiving a radiation dose less than 3000 rads with no other injury. The effects of various DRF's in the several scenarios differ widely and are affected by other hidden variables such as dispersion, accuracy of target acquisition and weapon delivery, and protective posture (shielding) at the time of attack. The gains in effective personnel are very significant in each scenario (compared to total casualties in Table 1) when the DRF is at least three. Even the radioprotective drugs with a DRF of only 1.5 had a definite beneficial effect.

In all three tactical scenarios, there appeared to be a significant tactical effect for DRF's of three or above, especially when short term troop effectiveness was considered. The experimental costs associated with achieving a DRF of three have not been assessed here and thus no overall conclusions on cost effectiveness of the research and development effort can be reached at this time. It should be pointed out that the RBE for the performance degradation studied here may be less than one and, since the DRF's were measured against the endpoint of lethality, the magnitude of the DRF's required for tactical significance may have been overestimated.

Table 1. Number of casualties in various initial radiation exposure bands (excluding those killed by blast effects)

DRF		INCA			Total Casualties
<u>Gamma</u>	<u>Neutrons</u>	<u>0-600 rads</u>	<u>0-1000 rads</u>	<u>0-3000 rads</u>	
*1	*1	736**	924	1668	7247
1.5	1.1	766	976	1789	7221
1.5	1.5	822	1100	2005	7104
3.0	3.0	1085	1487	2680	7065
5.0	5.0	1362	1874	3203	6941
TRANSANA LD 1D GAINS					
*1	*1	3995**	4308	4990	7584
1.5	1.1	4031	4344	5002	7417
1.5	1.5	4046	4364	5028	7388
3.0	3.0	4167	4482	5109	7077
5.0	5.0	4258	4548	5138	6852
TRANSANA LD 1A GAINS					
*1	*1	4137	5117	7324	14892
1.5	1.1	4260	5230	7398	14632
1.5	1.5	4403	5408	7594	14388
3.0	3.0	4866	5892	7897	13460
5.0	5.0	5149	6119	7990	12717

* No protective drug administered.

** Individuals receiving less than 150 rads and no other injury are considered non-casualties.

Table 2. Gains in effective personnel due to Dose Reduction Factors

		INCA			
DRF		No Effect (0-149 rads)	Non Lethal (150-599 rads)	Effective for hours (600-999 rads)	Effective for minutes (1000-3000 rads)
<u>γ</u>	<u>n</u>				
1.5	1.1	26	46	63	112
1.5	1.5	56	121	196	307
3.0	3.0	182	448	615	884
5.0	5.0	306	776	1011	1292

TRASANA LD 1D GAINS					
1.5	1.1	167	147	143	126
1.5	1.5	196	186	185	171
3.0	3.0	507	506	509	444
5.0	5.0	732	744	720	618

TRASANA LD 1A GAINS					
1.5	1.1	260	285	275	235
1.1	1.5	504	578	589	540
3.0	3.0	1432	1608	1806	1578
5.0	5.0	2175	2340	2286	1935

Project 3E162780A843 MEDICAL SYSTEMS IN CHEMICAL DEFENSE

Work Unit 002 Chemoprophylaxis of Ionizing Radiation Injury

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Project 3E161102BS09

BLAST OVERPRESSURE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTAINS SUMMARY DD FORM 1498-1	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8. DISSEM INSTR*	9. SPECIFIC DATA CONTRACTOR ACCESS	10. LEVEL OF SUM A. WORK UNIT	
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10. NO / CODES*	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER		
a. PRIMARY	61102A	3E161102BS09		00		001		
b. CONTRIBUTING								
c. CONTRIBUTING								
11. TITLE (Precede with Security Classification Code)*								
(U) Medical Effects of Blast Overpressure: Basic Studies								
12. SCIENTIFIC AND TECHNOLOGICAL AREAS*								
017100 Weapons Effects 002300 Biochemistry 016200 Stress Physiology								
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD		
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17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL & N YRS		20. FUNDS (in thousands)
a. DATES/EFFECTIVE: NA				PRECEDING				
b. NUMBER*				FISCAL		8		450
c. TYPE:				YEAR		CURRENT		
d. AMOUNT:				80		8		450
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NAME* Walter Reed Army Institute of Research				NAME* Walter Reed Army Institute of Research				
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				NAME: JAEGER, James, J., CPT, MSC				
				NAME: LORENZ, Patrick, CPT, MSC DA				
22. KEYWORDS (Precede each with Security Classification Code)								
(U) Blast Overpressure; (U) Acute Hemorrhagic Lung Injury; (U) Pulmonary Biochemistry; (U) Pulmonary Physiology; (U) Prostaglandins; (U) Thyroid Hormone Metabolism.								
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number - precede text of each with Security Classification Code)								
23. (U) To define the physiological effects upon the human of blast overpressure generated by firing Army weapons systems in terms of: (a) the physical characteristics of the pressure wave responsible for injury; (b) the interaction between the wave and susceptible organs; (c) the threshold for injury of the various organ systems; (d) potential means of prophylaxis and treatment of blast overpressure injury; and (e) the endocrinologic aspects of adaptive physiology.								
24. (U) Procedures include biochemical and physiologic measurements of pulmonary function and thyroid metabolism; biochemical methods include radioimmunoassay of prostaglandins, kallikreins, kinins, and thyroid hormone and measurement of coagulation and complement activation; physiologic methods include measurement of pulmonary tissue volume and capillary blood flow by re-breathing technique.								
25. (U) A canine model has been developed for the study of acute hemorrhagic pulmonary edema, induced by intravenous injection of oleic acid. Early results indicate that the initial event is focal thrombosis and hemorrhage into the lung, followed by progressive pulmonary fluid edema. Levels of prostaglandin degradation products rise markedly in arterial blood following oleic acid injection, suggesting the possibility that these metabolites may represent a marker of subclinical injury. A previously undescribed cytoplasmic enzyme involved is thyroid hormone metabolism - 5'-deiodinase - has been discovered in liver and kidney.								
For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sept 79.								

*Available to contractors upon originator's approval

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Project 3E161102BS09 BLAST OVERPRESSURE

Work Unit 001 Medical Effects of Blast Overpressure: Basic Studies

Investigators

Principal: MAJ Gary E. Sander, MC,
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Associates: CPT James J. Jaeger, MSC,
CPT Patrick Lorenz, MSC,
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CPT Jeffrey Hess, VC

Description

Studies are directed toward development of the basic sciences necessary to better evaluate the effects upon the human of blast overpressure generated by firing extended range Army weapons systems. This requires a detailed investigation of the biochemical and physiological changes occurring in the lung during acute injury in an attempt to describe markers of early, subclinical lung injury. The assessment of lung injury from any cause has been impeded by the absence of a sensitive indicator of early, developing injury. By the time that the chest roentgenogram has become abnormal and clinical symptoms are present, pulmonary edema is well advanced. All studies done to date examining blast induced injury in animals have utilized the microscopic appearance of the lungs at autopsy as the determinant of injury; thus the "threshold" of injury is defined as the lowest overpressure at which petechial hemorrhage appears in the lungs. The ability to detect earlier stages of injury is absolutely dependent upon the detection of biochemical or physiological markers reflecting the pathophysiological alterations in the lung. Such markers would also permit assessment of the evaluation, progression, and resolution of injury, as opposed to present autopsy studies which allow evaluation of only a single point during the time course of injury.

In order to address these issues, the research effort described in WRAIR Research Protocol (005-79) "Physiological and Biochemical Assessment of Acute Hemorrhagic Pulmonary Edema in the Dog" is now underway. Acute hemorrhagic lung injury is produced by intravenous injection of oleic acid, and kinins, prostaglandins, and coagulation parameters assayed in arterial

and venous blood as potential markers of injury. Pulmonary tissue volume and capillary blood flow will be measured to determine their ability to adequately quantitate early injury. This protocol is currently in progress, and since results are still incomplete, only the development of the model, methodologies for assay of the biochemicals, and instrumentation and methodology for the rebreathing technique will be described in this report.

Many hormonal alterations are known to occur in a variety of pathophysiologic conditions; these changes may occur in the hypothalamus, pituitary, and thyroid glands and in peripheral tissues. Studies are currently directed toward examining the effects of various stresses upon: a) the peripheral metabolism of iodothyronines (thyroid hormones and their metabolites) and, b) the hypothalamic-pituitary control of these activities. These results are summarized.

Progress

1. Physiological and Biochemical Assessment of Acute Hemorrhagic Pulmonary Edema in the Dog.

a. Canine Model: A canine model was selected because the dog is of sufficient physical size to allow reasonable ease of placement of the required catheters and because the dog has an adequate blood volume to allow removal of the required blood samples without inducing hypotension. General anesthesia is induced with pentobarbital sodium (35 mg/kg) and respiratory paralysis with pancuronium bromide (0.25 mg/kg). Additional doses of each are administered at 30 minute intervals to maintain adequate anesthesia and to suppress spontaneous respiratory activity. A Swan-Ganz thermodilution catheter is placed into a wedge position in the pulmonary artery via the right external jugular vein; additional catheters are placed in the right internal carotid artery, and the inferior vena cava via the right femoral vein. These catheters are flushed with saline as necessary to maintain potency. The Swan-Ganz catheter is utilized for monitoring pulmonary artery and pulmonary arterial wedge pressures, and thermodilution cardiac outputs, the superior vena caval catheter for monitoring central venous pressures and obtaining venous blood samples, the arterial catheter for monitoring arterial pressures and obtaining arterial blood samples, and the inferior vena caval catheter for injection of oleic acid.

The trachea is intubated with a cuffed endotracheal tube and

the animal ventilated with room air by a constant volume ventilator. Tidal volume is set at 15 ml/kg and the rate adjusted to maintain end-tidal P_{CO_2} at 40 Torr. Inspired and expired gas concentrations are monitored continuously by an eight-channel mass spectrometer via a line attached to the tracheal cannula. The electrocardiogram, central venous pressure, pulmonary artery pressure, systemic arterial pressure, airway pressure, and ventilatory O_2 and CO_2 concentrations are recorded continuously by a multi-channel² strip chart recorder. Thermodilution cardiac output is measured every 30 minutes. Arterial and venous blood samples are obtained simultaneously every 30 minutes for measurement of prostaglandins, kinins, and coagulation parameters.

At the end of data collection for a particular dog, the chest is opened, inferior and superior vena cava ligated, the trachea cut 2 cm above the carina and the lungs removed. After severing all connecting vessels and tissue at the hilum, blood is drained passively from the lungs. The lungs are then weighed, frozen in liquid nitrogen, and freeze dried to constant weight. The lungs are homogenized and an aliquot of the homogenate assayed for Fe content by atomic absorption spectroscopy. This Fe content is then utilized to determine the blood content of the lungs and to correct wet and dry weights accordingly.

b. Oleic Acid Induced Hemorrhagic Pulmonary Edema:

Oleic acid, an 18-carbon mono-unsaturated free fatty acid, produces hemorrhagic pulmonary edema when injected intravenously, with extent of injury proportional to the dose. Low doses produce little clinical evidence of injury but macroscopic injury is present in the lungs in the form of irregularly distributed hemorrhagic lesions which progress into ventral and subplural areas of consolidation (1). Higher doses will produce severe injury characterized by tachypnea, decreased lung compliance, and hypoxemia with death within several hours. The lungs of these animals show severe hemorrhagic edema, vascular congestion, and atelectasis (2).

Histologically, edema and capillary congestion become evident within one hour after injection; alveolar edema and hemorrhage and septal necrosis are present within 6 to 12 hours. These findings are less evident by 24 hours and except for moderate edema, are not present after one week. After 2 to 4 weeks, areas of interstitial and septal fibrosis are present. The mechanism by which oleic acid produces these lesions is suggested by the electron microscopic data. Early capillary congestion appears to be caused by thrombi containing fibrin, platelets, and cell debris. At this time endothelial and type I alveolar

cells appear necrotic and are separated from the basement membrane. Four hours post injection, capillary obstruction is no longer evident but endothelial cells show increasing abnormalities for the next 20 hours. The cellular events occurring after 24 hours reflect a proliferation of type II cells and an increase in collagen (2).

In our model an oleic acid dose of 35 mg/kg was found to produce the desired injury level at two hours with 30-50% reduction in P_{O_2} and approximately a two fold increase in the ratio of wet lung to whole body weight. At autopsy the lungs show consolidated areas of hemorrhage located primarily on ventral surfaces. Histologic evaluation done 2 hours after oleic acid revealed a sub-plural vasculitis with some degree of alveolar edema and fibrin deposition. There was no evidence of intravascular thrombosis, and no extra pulmonary evidence of injury. Interestingly, dogs autopsied 30 minutes after oleic acid displayed consolidated and focal hemorrhages almost indistinguishable in appearance from the 120 minute dogs. However, the lung wet weight to whole body ratios were normal to only slightly elevated, suggesting that the oleic acid induced injury begins as local hemorrhage without edema with subsequent slow evolution of fluid edema beginning between 60 and 90 minutes. This corresponds with the arterial blood gas measurements, which reveal falling P_{O_2} during the same time frame.

Higher doses of oleic acid, 70 mg/kg and 100 mg/kg, produced much more severe injury, with bloody froth in the endotracheal tube within 90 minutes and severe hypoxemia (P_{O_2} =40 torr). Death occurred within 120 minutes from hypoxemia and severe acidosis.

c. Biochemical Markers: Three classes of biological molecules are under investigation as potential markers of early lung injury: vasoactive peptides, prostaglandins, and coagulation components. A single enzyme located on the endothelial cell plasma membrane - angiotensin I converting enzyme or kininase II - is capable of inactivating the nonapeptide bradykinin and converting the inactive decapeptide angiotensin I to the vasoconstrictor octapeptide angiotensin II during passage through the pulmonary circulation (3). Disruption of the endothelial cell might be expected to alter the metabolism of these peptides. Bradykinin is also formed and released during both local and systemic (anaphylaxis) injury. Bradykinin is formed in plasma from kininogen by the enzyme kallikrein; kallikrein is itself activated from prekallikrein by activated

Hageman factor. In order to adequately categorize the bradykinin system, it is necessary to measure both kallikrein and bradykinin. Our laboratory has developed a significant improvement over existing bradykinin radioimmunoassays which require multiple chromatography steps and result in recoveries often as low as 60% (4). This newly developed methodology requires a single step separation of bradykinin from plasma on Saphadex G-25 PD-10 columns, with a bradykinin recovery of 85%, and with a lower limit of sensitivity of 25 pg/ml. Utilizing this assay, 20-30 plasma samples may be totally assayed within 6 hours (5). Kallikrein is measured by use of the synthetic substrate TAME (tyrosyl-arginyl-methyl (³H)-ester), which is converted to tyr-arg + methanol (³H). ³H methanol is then counted as an index of kallikrein activity.

The angiotensin I converting enzyme is present in plasma as well as lung tissue. Although the plasma enzyme is much less active than the lung endothelial system (6), a recent study suggests that both enzymes behave identically after purification (7). The only discernible difference between the two enzymes was in carbohydrate content. Thus the plasma enzyme may well originate from the pulmonary endothelial cell and the level of the plasma enzyme may thus reflect endothelial injury. Angiotensin I converting enzyme activity will be assayed utilizing the synthetic substrate (³H) hippuryl-histadyl-leucine; the enzyme cleaves his-leu from ³H-hip, and thus ³H-hip may be separated and counted as an index of converting enzyme activity.

The prostaglandins are a complex family of acid lipids which mediate a wide variety of physiological events (8). Parent prostaglandins of the E and F series entering the pulmonary circulation are effectively removed from the blood by active transport across the endothelial cell membrane, where they are both metabolized and stored. They are also synthesized within the lung, and released into the systemic circulation in response to a variety of insults such as anaphylaxis and pulmonary edema. The prostaglandins PGE₂ and PGF₂ are metabolized to 13,14-dihydro -15-keto degradation products. Large quantities of the degradation products, but not of the parent prostaglandins, are released during anaphylaxis (9). These 13,14-dihydro -15-keto products have a half life in blood of 8½ minutes, and are further metabolized primarily in the liver (10). Thus these degradation products provide a relatively stable index of prostaglandin metabolism. Our laboratory now has functional radioimmunoassays for prostaglandins F_{2a}, 13,14-dihydro -15-keto F_{2a}, and 13,14-dihydro -15 keto-E₂^a, with

a range of sensitivities from 5 - 10,000 pg. The antibody for the E_2 metabolite cross-reacts fully with that of the F_{2a} , and thus it can be used to detect the combined metabolites of F_{2a} and E_2 . Early results of this protocol show marked elevations of these combined metabolites within 30 minutes of oleic acid infusion.

Minimal endothelial injury results in local platelet aggregation and fibrin deposition (11). with activation of coagulation probably secondary to high concentrations of thrombo plastin on the plasma membrane of the endothelial cell (12). It has been suggested that complement activation may also occur during coagulation. Activated C3 is covalently linked to leukocytes and enzthrocytas as they circulate past sites of injury; thus if blood cells can be demonstrated to acquire C3 during passage through the lungs, this would be strong evidence of early injury. Arterial and venous platelet counts and coagulation profile (to include fibrin split products) will be measured, as will total serum complement by the hemolytic method using sheep erythrocytes sensitized to the classical pathway of complement activation. If this assay shows evidence of complement activation across the pulmonary circulation, a more sensitive technique searching for C3 covalently linked to enzthrocytes or leukocytes will be utilized. C3 binding will be measured using fluorescein or rhodamine labeled antibody to C3.

d. Pulmonary Function Testing: Pulmonary tissue volume (V_T) and pulmonary capillary blood flow (\dot{Q}_C) are measured by a soluble gas uptake technique involving the rebreathing of a helium, acetylene, oxygen and nitrogen gas mixture (13). V_T is defined as the volume of tissue and fluid which absorbs acetylene from airways during the first breath of the rebreathing period. Thus, V_T represents the septal tissue of the lung and has been shown to be sensitive to varying degrees to interstitial pulmonary edema. \dot{Q}_C is defined the rate of blood flow thru capillaries in ventilated lung regions which accounts for the constant rate of disappearance of acetylene from the lung-bag system during the 15 to 20 second rebreathing period. Because of anatomic and physiologic shunting of blood away from ventilated regions of the lung, \dot{Q}_C is a variable fraction of the cardiac output which is on the order of 10% in normal dogs.

Preliminary inspection of rebreathing data collected to date has shown that the technique as applied in our laboratory produces results comparable to those in the literature

(Table 1). This data was summarized from the $t=0$ min measurement period and thus represents control values for healthy, anesthetized, paralyzed and ventilated dogs lying in the supine position. Additional analyses are on-going to determine the relationship of V_T and \dot{Q}_C to developing oleic acid-induced lung injury as well as the relationship between V_T and post-mortem lung weights.

e. Experimental Design: In order to determine the relationship between the variables measured by both the rebreathing technique and the biochemical assays and developing pulmonary edema, animals will be sacrificed at 30, 60, 90 and 120 minutes post oleic acid injection. Four animals will be needed for each of the above conditions; an additional four animals will be sacrificed at time 0, without receiving an injection of oleic acid. Arterial and venous blood samples, arterial blood gases, thermodilution cardiac output, and rebreathing measurements will be performed every 30 minutes, beginning 30 minutes before oleic acid infusion.

2. Endocrinologic Studies

a. Peripheral Metabolism of Iodothyronines: In the past 3 years, many studies have documented that thyroxine may be converted peripherally (primarily in liver and kidney) to either T3 (metabolically very active) or reverse T3 (essentially inactive), depending upon whether deiodination occurs at the phenolic or tyrosyl ring. This process is enzymatic, and it is now known that metabolism is altered by a variety of stresses including starvation, high dose glucocorticoids, acute medical illnesses (including malaria), chronic diseases, surgery, severe burns, and a number of pharmacologic agents. Interestingly, in all these instances the organism markedly decreases conversion to T3 and increases conversion to reverse T3. One might think this alteration in metabolism would be protective, since it diverts the body away from production of a catabolic, active hormone (i.e. T3) to an inactive hormone (rT3). Limited kinetic studies indicate a more complex process is involved. Moreover, it is unclear whether this shift in iodothyronine metabolism is always beneficial since in certain circumstances it appears to predict a fatal outcome.

T3 and rT3 may be further deiodinated (14) to diiodothyronines (3', 5' T2; 3, 3' T2; and 3, 5 T2) and moniodothyronines (3' T1; 3 T1), and development of sensitive and specific radioimmunoassays for these iodothyronines (15-17) has permitted investigations into the effects of various pathophysiologic states on the pathways of deiodination.

Most studies investigating peripheral deiodination have assessed T4 to T3 conversion in vitro in rat liver homogenates or microsomes. This process involves deiodination at the 5' position, and the putative enzyme has been designated "5'-deiodinase". There are 2 other substrates that also may be deiodinated at the 5' position, these being rT3 and 3',5'T2, and several laboratories have shown that rT3 to 3,3'T2 conversion behaves similarly to T4 to T3 conversion. This has led to the speculation that there is only one 5'-deiodinase (localized to microsomes). However, using a radioimmunoassay (RIA) developed in this laboratory (16), we have been able to evaluate 3',5'T2 to 3'T1 conversion in vitro. Fully expecting this reaction to behave identically to the other 2 reactions for 5'-deiodination, it came as a surprise when significant cytosol enzymatic activity was discovered. A series of experiments during the past year has determined that indeed there may be more than one 5'-deiodinase, as the enzyme activities in microsomes and cytosol have different Kms of 8.6×10^{-9} M and 2.4×10^{-7} M, respectively (18). Moreover, the changes reported for T4 to T3 and rT3 to 3,3'T2 conversion during hyper- and hypothyroidism and during fasting are not identical to those for 3',5'T2 to 3'T1 conversion (19). This was first recognized in homogenates, and we have recently extended our observations to demonstrating that during fasting conversion of T2 to T1 is not inhibited in cytosol, whereas it is in microsomes. The significance of more than one enzyme is not readily apparent at the present time, especially since the major 5'-deiodination of metabolic importance (i.e. T4 to T3) seems to be regulated by but one enzyme. However, it may be that an additional "scavenger" enzyme is important in more efficiently deiodinating these inactive metabolites so that iodide can be recycled for the production of new hormone.

Current and projected studies include development of an RIA for 3-monoiodothyronine (the only iodothyronine yet to be identified), in vitro studies tyrosyl ring deiodination, dose response studies of pharmacologic agents that affect thyroid hormone metabolism, and purification of the enzyme(s) involved.

b. Human Kinetics: A second area of interest is the kinetics of iodothyronines in humans. The metabolic clearance rate (MCR) and production rate (PR) of reverse T3 in patients with hyper- and hypothyroidism was recently reported (20). This study was done using a bolus injection of 125 I-rT3, similar to the method used for determining T4 and T3 kinetics. Extending these studies to 3',5'T2, we have learned that this hormone is so rapidly metabolized (even during the study) that a constant function is necessary, and that serum samples

must be processed on a G-25 Sephadex column to isolate the T2 fraction. A new concept emerging from this study pertains to doing the infusions with unlabelled hormone rather than ¹²⁵I-hormone and measuring serum concentrations by RIA. This method has never been applied to iodothyronines, so infusions of both labelled and unlabelled hormone (separate days, same patients) have been performed and the preliminary results indicate the 2 methods are comparable (21). It is advantageous to establish that use of unlabelled hormone is valid, both because of avoiding the use of isotope and because of the ease of processing samples.

Current investigations involve the study of other stresses which influence 3',5'T2 metabolism, and also kinetic studies of 3,3'T2.

c. Thyroid Hormone Receptors: The initial site of action of thyroid hormone appears to be localization of T3 (and possibly T4) at intranuclear receptor(s). While receptors have been demonstrated in many tissues in the body, most studies have used rat liver nuclei because of technical considerations. The liver may not reflect the effect of thyroid hormone on other target organs, however, and we have some preliminary data suggesting that T3 receptors in the heart are altered in a manner differently than in liver in hyper- and hypothyroid rats (22-23).

d. Hypothalamic-Pituitary Disorders: Using starvation as a stress model, alterations in both the secretion and synthesis of thyrotropin (TSH) from the pituitary gland have been demonstrated during a prolonged infusion of thyrotropin releasing hormone (22-23). Changes in glucogen kinetics during fasting also appear to be regulated, at least in part, by alterations in serum T3 (24).

TABLE 1

Rebreathing Data Summary. Control Values.

Variable	Present Study	Peterson et al (13)
BW (kg)	22 \pm 3 (20)	19 \pm 3 (20)
V_T (ml)	233 \pm 63 (20)	215 \pm 51 (20)
\dot{Q}_C (L/min)	2.7 \pm 0.6 (20)	2.5 \pm 0.6 (20)
V_T /BW (ml/kg)	11	12
\dot{Q}_C /BW (ml/min/kg)	114	132
CO	3.5 \pm 1.0 (14)	3.8 \pm 1.0 (15)
\dot{Q}_C /CO	74%	66%

BW= Body Weight; V_T = pulmonary tissue volume; \dot{Q}_C = pulmonary capillary blood flow; CO= cardiac output determined by thermal dilution (present study) or by dye dilution (Peterson et al) during tidal breathing immediately before rebreathing procedure. Values are means \pm SD with the number of animals in parentheses.

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